Revealing *Manduca sexta*'s nicotine metabolism and its ecological consequences using plant mediated RNAi based reverse genetics

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von

Pavan Kumar

geboren am 17. September 1982 in India

Gutachter:

Prof. Dr. Ian Thomas Baldwin, Max-Planck Institute for Chemical Ecology, Jena, Germany

Prof. Dr. David Heckel, Max-Planck Institute for Chemical Ecology, Jena, Germany

Prof. Prof. Birger Lindberg Møller, University of Copenhagen, Denmark

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Chapter 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

1.1 Background

Plants are exposed to myriad of biotic and abiotic stresses during their growth and development. Herbivory is one of the main threats to plants, since herbivores can defoliate a complete plant and destroy plant's reproductive structures. Although insects are the most common herbivores, plants and insects co-exist in the environment since several million years (Engel & Grimaldi 2004). One of the main reasons of plants being successfully evolved is their endogenous, sophisticated and fine-tuned 'defense system', which can thwart growth and development of insect herbivores. Plants possess structural and chemical layer of defense system against insect herbivores. Leaf toughness, epidermis and trichome development are the structural barriers (Levin 1973; Cooper & Owensmith 1986; Eisner *et al.* 1998; Lev-Yadun 2003). Chemical defenses include volatiles, toxins (nicotine, cardiac glycosides), antinutritive (phenolics) and antidigestive compounds (proteinase inhibitors) (Baldwin 1988; Zavala *et al.* 2004; Bednarek & Osbourn 2009).

Insects are considered to be one of the most successful organisms on this planet (http://www.ucmp.berkeley.edu/arthropoda/arthropoda.html). Herbivorous insects have been constantly exposed to myriad of plant defense metabolites and they have developed highly effective counter-defense systems (Bernays & Graham 1988). They have adapted to counter the plant defense metabolites by detoxification, avoidance, sequestration and rapid excretion of ingested plant allelochemicals. In addition, insects have to defend against their natural enemies such as predators and parasitoids (Bentz & Barbosa 1990; Wink 1993; Brown KS 1995).

1.2 *Nicotiana attenuata- Manduca sexta*

N. attenuata (Solanaece) is a tobacco plant native to Great Basin desert-Utah, USA **(Fig 1)**. *N. attenuata* germinates post fire in a nitrogen rich soil and grows under a highly competitive environment (Baldwin & Morse 1994). *N. attenuata* encounters a several herbivores such as mirids (*Tupiocors notatus*), empoasca grasshoppers,

negrobugs, lepidopteran generalists (*Spodoptera exigua* and *Heliothis virescens*) and specialists (*M. sexta* and *M. quinquemaculata*) **(Fig 2)**. *M. sexta,* commonly known as hawkmoth or hornworm or sphinx moth is a major herbivore of *N. attenuata*. *Manduca* moth lays eggs underneath *N. attenuata* leaves; after hatching, neonates start to feed on the leaf trichomes and eventually on leaves, until the beginning of pupation event known as wandering stage. *M. sexta* larvae pass through five different instars/ molting stages to reach pupation and later to emerge as a moth (http://insected.arizona.edu/manduca/web_ resources.html).

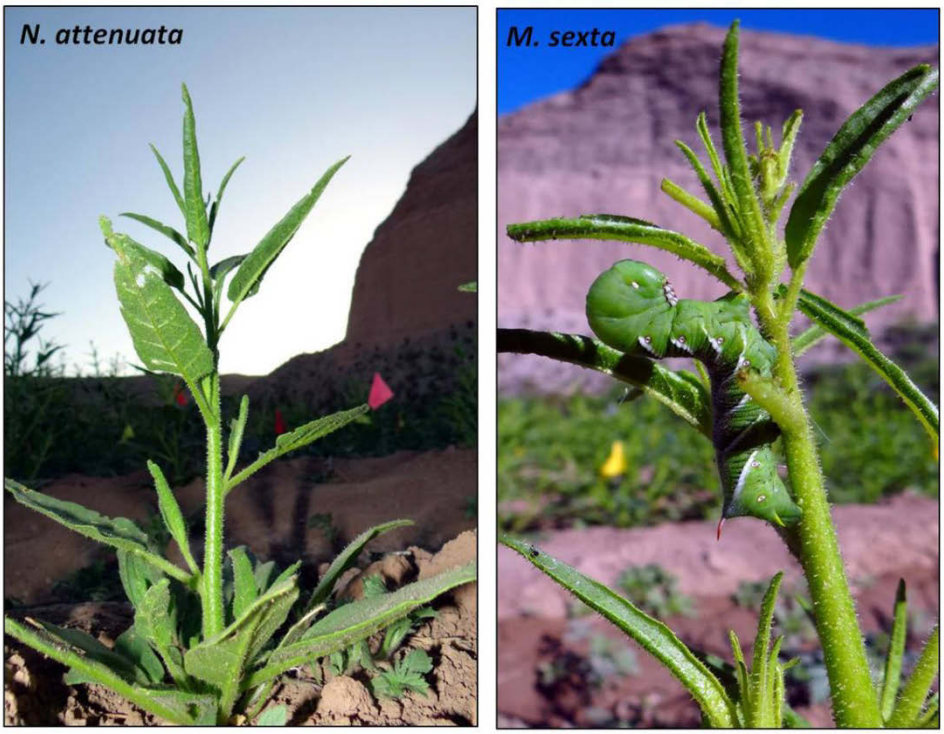

Fig. 1 Ecological model plant *N. attenuata* (left) and its specialist herbivore *M. sexta* (right) at their native habitat Great Basin desert, Utah (USA).

N. attenuata responds to *M. sexta* attack in a fine-tuned manner. The defense system of *N. attenuata* against the specialist herbivore *M. sexta* is well studied. *N. attenuata* uses a direct and indirect defense response to *M. sexta* attack. *M. sexta*'s oral secretion triggers the defense response. Fatty acid-amino acid conjugates (FACs) are the key players, which are responsible for this induction (Halitschke *et al.* 2001). Direct

defense response includes production of insect digestive enzyme (protease) inhibitors, antinutritive diterpene glycosides and an alkaloid nicotine (Baldwin 1988; Steppuhn *et al.* 2004; Zavala *et al.* 2004; Jassbi *et al.* 2008). In case of indirect defense response, plants emit volatile organic compounds (VOCs), which in turn attract the predators of herbivore insects (Dicke 1994; Kessler *et al.* 2004).

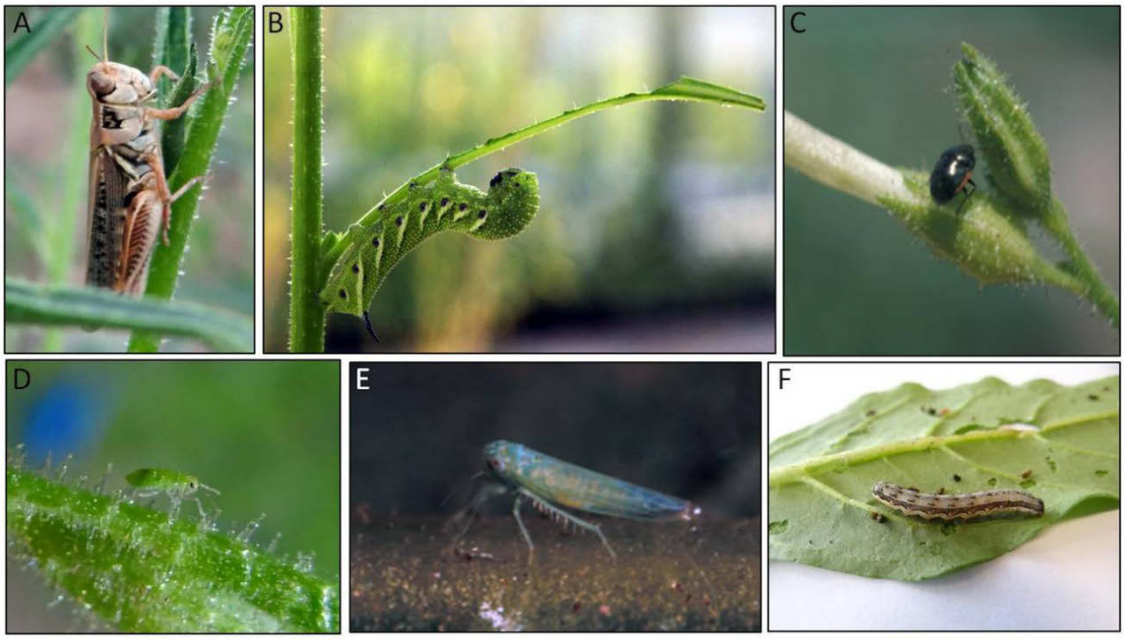

Fig. 2 Native herbivore community of *N. attenuata*

(A) grasshopper (B) hornworm (C) negro bug (D) aphid (E) leafhopper and (F) beet armyworm.

1.3 Nicotine

Nicotine, a pyridine alkaloid is the most abundant metabolite in tobacco plants and it is one of the early alkaloids used to control insect pests (Schmeltz 1971; Wink 1993). Nicotine acts as a major direct defense metabolite of tobacco plants. It has been already established that *M. sexta* larval attack, induces a nicotine accumulation in *N. attenuata* leaves (Baldwin 1988). Nicotine affects *M. sexta* larval performance in nature. Larvae feeding nicotine-less plant [silenced for putrescine N-methyl transferease (irPMT) transcripts accumulation] grow faster than those feeding wild type (WT) *N. attenuata*

(Steppuhn et al. 2004). Nicotine acts on nervous system of insects by mimicking the neurotransmitter acetylcholine, thereby affecting the acetylcholine mediated signals. However, *M. sexta'*s tolerance to nicotine is higher than that of any other organism (Wink & Theile 2002). Hence, understanding the molecular mechanism behind this remarkable nicotine tolerance of *M. sexta* is important.

1.4 Nicotine detoxification by *M. sexta*

M. sexta has been used as an insect model for nicotine tolerance and/ or detoxification research due to its exceptional nicotine tolerance strategy (Wink & Theile 2002). Three lines of theories have been proposed to explain *M. sexta*'s nicotine tolerance: Firstly, cytochrome P450s (CYPs) mediated oxidation to less toxic forms nicotine-N-oxide (NNO), cotinine and cotinine N-oxide (CNO). Synder et al report that nicotine ingestion induces midgut based CYPs belonging to the family 4 such as CYP4M1 and CYP4M3. These CYPs were hypothesized to be involved in oxidation of nicotine (Snyder *et al.* 1993; Snyder *et al.* 1994; Snyder *et al.* 1995a). Oxidized forms of nicotine were thought to be less toxic and due to their increased polarity nature than nicotine, nicotine oxides were thought to be excreted faster than nicotine (Hodgson 1985; Snyder *et al.* 1994). Secondly, rapid excretion of nicotine without modification. Self et al reported that there was no metabolism of nicotine in *M. sexta*; ingested and injected nicotine was excreted as unchanged nicotine in frass. Self et al did not find any oxidized products of nicotine in larval hemolymph or frass (Self *et al.* 1964a; Self *et al.* 1964b). Maddrell and Gardiner supported the 'no metabolism' by demonstrating the presence of alkaloid pump in Malpighian tubules that help nicotine excretion (Maddrell & Gardiner 1976). The third line of explanation of *M. sexta*'s nicotine tolerance is that its nerve cells have become less sensitive to nicotine (Morris 1984). None of these theories have been supported by sufficient evidence (Appel & Martin 1992; Kumar *et al.* 2013).

1.5 Cytochrome P450s

Cytochrome P450s belong to a multigene family and they derive the first part of their name 'CYP' from the presence of a heme factor 'cytochrome b5' in them and the

second part 'P450' reffered, since they absorb maximum light at 450 nm in presence of CO. CYPs catalyze diverse chemical reactions such as oxidation, epoxidation and hydroxylation reactions. The most common reaction catalyzed by CYPs is addition of one oxygen atom to an organic substrate (Berge *et al.* 1998; Feyereisen 2005; Feyereisen 2011). CYPs are the key players of insects' xenobiotic detoxification systems.

Induction of CYPs in response to xenobiotics or plant allelochemicals is already well known in insects. *M. sexta* CYPs of family 9 were reported to be induced in response to various xenobiotics (Scott 1999). Snyder et al reported the induction of CYP4M1 and CYP4M3 in response to dietary nicotine (Snyder *et al.* 1993; Snyder *et al.* 1995a). Govind et al studied the 24h old *M. sexta* neonate's midgut transcriptional response to dietary nicotine by microarray analysis. They found that *Ms*CYP6B46 was induced in response to nicotine ingestion (Govind *et al.* 2010). Often CYPs belong to the family 6 were found to be involved in xenobiotic detoxification. We hypothesized that CYP6B46 is involved in the detoxification of nicotine. We tested the role of CYP6B46 by silencing it. Further, we used larval hemolymph and frass for the qualitative and quantitative analysis of nicotine and nicotine metabolites. In addition, overall changes in the nicotine flux were calculated with the help of classical Waldbauer assay. It is important to know the function of such candidate genes, from induction and microarray based transcriptome data, to unveil ecological interactions.

1.6 RNA interference

RNA interference (RNAi) is an endogenous mechanism of eukaryotic cells to control the gene regulation. RNAi was first successfully demonstrated in *Caenorhabditis elegans* (Fire *et al.* 1998). RNAi process is triggered by the endo- or exogenous long dsRNA molecule having the sequence similarity with target mRNA. Such a long dsRNA molecule is cleaved into small RNA duplexes (~21 to 24 nt) by Dicer, a ribonuclease III (RNase III) enzyme in an ATP dependent reaction. Different types of Dicers have been reported and they are known for generating different types of RNA molecules such as small interfering RNA (siRNA), miRNA etc. In *Drosophila melanogaster*, Dicer-1 is reported to produce miRNAs and Dicer-2 produces siRNAs (Lee et al., 2004). The

resulted molecules were then incorporated into a multiprotein complex known as RNA-induced silencing complex (RISC). The RISC complex generates a guide strand and finds potential target messenger RNAs (mRNA) based on complimentary sequences. Later, Argonaute (Ago) protein, an endonuclease bound to RISC cleaves the target mRNA molecule leading to downregulation of the target mRNA accumulation. The efficiency of silencing a gene is depends upon the amplification of the silencing signal. Generation of subsequent siRNA molecules, known as secondary siRNA molecules by RNA dependent RNA polymerase (RdRP) enhances the silencing efficiency. Surprisingly, there is no clear evidence of the presence of RdRP activities in lepidopteran insects. In addition, silencing efficiency also depends upon the systemic RNA interference deficient-1 (*sid-1*) gene. Sid1 is responsible for the spreading of the silencing signal systemically (Winston *et al.* 2007).

Different types of methods of introducing the dsRNA molecules into insects, such as feeding a droplet, through diet or plant, injection and soaking, have been reported (Yu *et al.* 2013). Although injection of dsRNA is often practiced, the major limitation of this technique is lack of continues supply of dsRNA molecules. There are increasing reports of gene silencing in lepidopteran insects including *M. sexta* (Eleftherianos et al. 2006; Garbutt et al. 2013). Genes involved in immunity have been the main targets and injection has been the method of choice of dsRNA delivery (Eleftherianos *et al.* 2006; Zhu 2013). However, there were no reports of PMRi in *M. sexta* before we could publish our results (Kumar *et al.* 2012). Mao et al demonstrated a host plant mediated continuous supply of dsRNA molecules into insect midgut (Mao *et al.* 2007). They demonstrated *Arabidopsis thaliana* mediated silencing of *Helicoverpa armigera CYP6AE14* gene, which is responsible for providing a tolerance against gossypol. They emphasized the role of host plant as food as well as a source of double stranded RNA (dsRNA). They showed that while feeding, the dsRNA produced by stable transgenic host plant could penetrate the gut cells of insect and could reduce the expression of target gene by post transcriptional gene silencing (PTGS). This strategy enabled the study of insect gene function during ecological interactions, in ‘real time’.

We used PMRi to silence *M. sexta*'s CYP4M1, CYP4M3 and CYP6B46 genes (Kumar et al). We developed a transgenic *N. attenuata* plant expressing *Ms*CYP6B46 dsRNA; since this plant was transformed with an expression vector containing ~300bp fragment of *Ms*CYP6B46 cDNA in an inverted repeat (ir) manner, we called these plants as irCYP plants **(Fig 3)**. In addition, to PMRi, we also established a transient, plant virus based dsRNA producing system (VDPS) to silence *Ms*CYP4M1 and *Ms*CYP4M3. VDPS is a reframed form of Virus Induced Gene Silencing (VIGS) technique used for silencing the plant genes (Saedler & Baldwin 2004). In VDPS, tobacco rattle virus infected *Agrobacterium tumefaciens* is infiltrated into *N. attenuata* plants to express the dsRNA of insect gene. Development of PMRi and VDPS and successful demonstration of silencing *M. sexta* larval genes is described in manuscript-I.

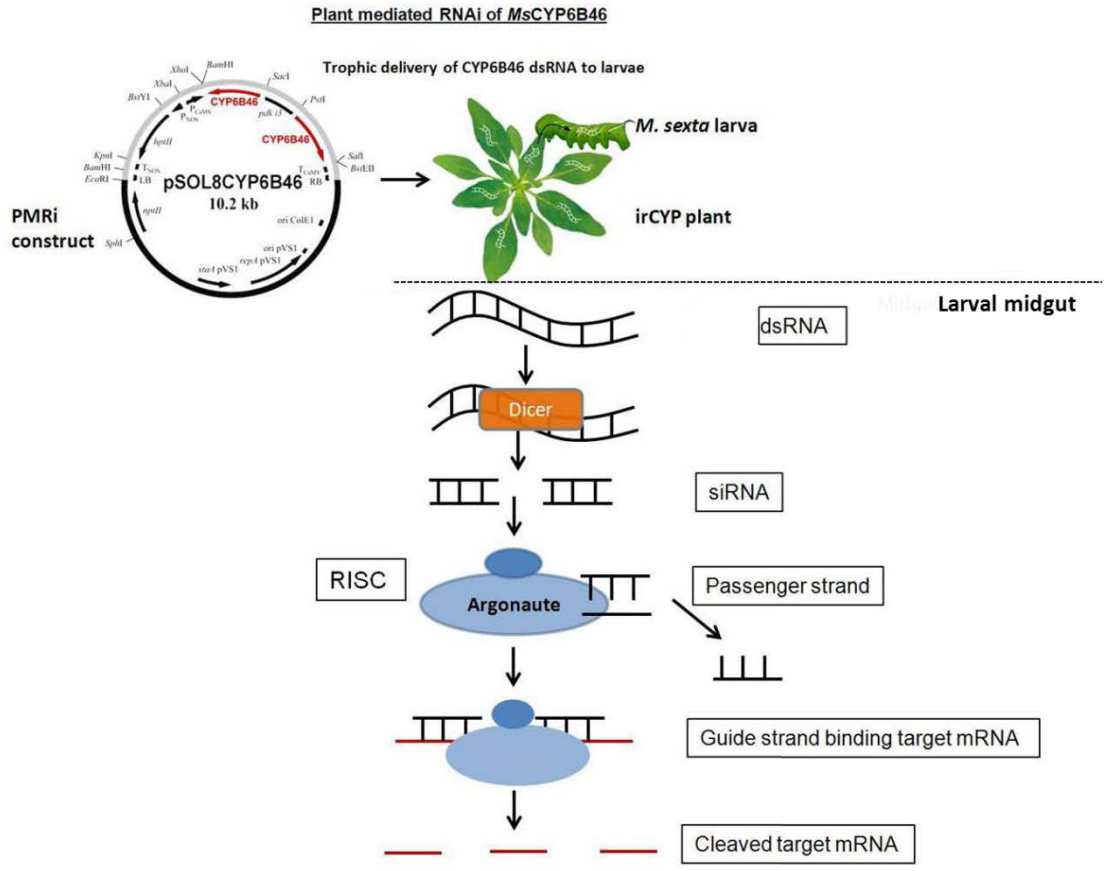

Fig. 3 Plant mediated RNAi (PMRi)

Schematic hypothetical representation of plant mediated RNAi; pSOL8 binary vector is Agro-transformed into *N. attenuata* plant to express ~300bp long dsRNA of *Ms*CYP6B46, which is later tropically transferred from plant to larvae. dsRNA penetrates into insect midgut cells and by the catalytic activity of putative dicers, dsRNA is processed and resulred smRNA/ siRNA molecules are recruited by RISC complex (not characterized in lepidopterans). The RISC complex generates a guide strand and finds the target mRNA based on sequence homology with the help of Ago proteins and subsequently degrade the target mRNA.

1.7 Chemical interactions between *N. attenuata* and *M. sexta*

Native habitat of *N. attenuata* and *M. sexta* is hovered with various predators **(Fig 4)**. *M. sexta* is constantly under threat of a predator community in the field. Big eyed bug, *Geocoris pallens*, is a major predator in the field, in addition to ants, ant lions, praying mantes, lizards and spiders. However, there is no clear knowledge of molecular or chemical interactions between *M. sexta* and these predators. Although it is known that, *M. sexta* larvae efficiently excrete nicotine and a proficient blood-brain barrier is involved, its nervous system is exposed to nicotine levels that are lethal to unadapted insects (Morris 1984; Murray *et al.* 1994). Hence, within the limits of *M. sexta*'s excretory-based tolerance lie opportunities for the defensive use of nicotine, particularly against endoparasitoids, which develop within larval tissues. Indeed it was reported that more *Cotesia congregata* endoparasitoids emerged as adults from parasitized *M. sexta* larvae fed on low nicotine varieties of cultivated tobacco than from larvae fed nicotine-rich varieties (Thorpe & Barbosa 1986). The generalist predatory ant *Iridomyrmex humilis* also preferred *M. sexta* larvae reared on artificial diets (AD) without nicotine over those reared on high nicotine diets and were deterred by topical nicotine treatments as well (Cornelius & Bernays 1995). Thus *M. sexta* larvae might be able to use this diet-derived toxin for its own protection by virtue of its tolerance machinery, despite a lack of clear sequestration and storage of this toxin. *M. sexta* larval adaption for host plant toxin, nicotine and role of nicotine-induced CYP6B46 in larval nicotine physiology and ecology is described in manuscript II.

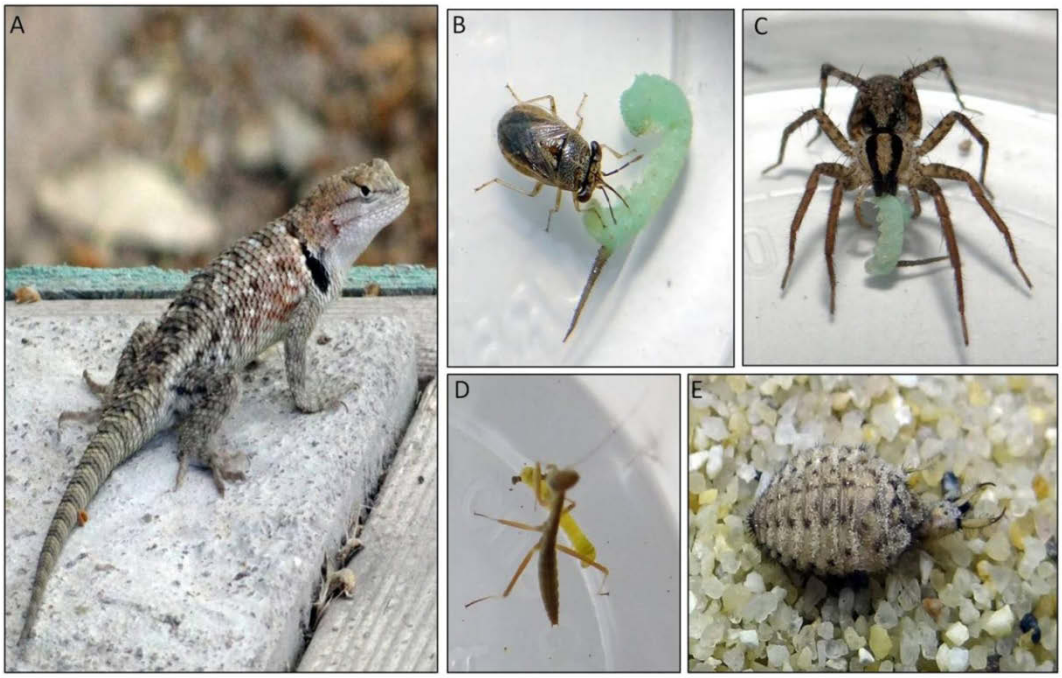

Fig. 4 Native predators of lepidopteran herbivores

Common predators of lepidopteran herbivores native to the Great Basin desert, Utah (USA) (A) spiny lizard (B) big eyed bug (C) wolf spider (D) praying mantis and (E) ant lion.

Studying generalist-specialist paradigm has always been exciting to the ecologists. It is already well known that plants perceive and respond to generalist and specialist herbivores differently (Vogel *et al.* 2007). On the other hand, generalist insect's strategy against host plants' allelochemicals is different than the specialist (Ali & Agrawal 2012). Hence, it is interesting to know how generalist insects deal with host plant toxins and their natural enemies. *S. exigua* and *H. virescens* are common lepidopteran generalist herbivores of *N. attenuata*. Nicotine detoxification strategy of generalist (*S. exigua*) and specialist insect (*M. sexta*) herbivores, and its physiological and ecological consequences are described in manuscript III.

Overall objective of the present was to revealing the midgut based nicotine induced *Ms*CYP6B46 role in nicotine detoxification and adaptation by reverse genetics approach. Secondly, to study the ecological consequences of nicotine detoxification and adaptation by specialist insect *M. sexta* and generalist insect *S. exigua.*

Chapter 2

OVERVIEW OF MANUSCRIPTS

MANUSCRIPT I

Tobacco rattle virus vector: A rapid and transient means of silencing *Manduca sexta* genes by plant mediated RNA interference

Pavan Kumar, Sagar Subhash Pandit, Ian T Baldwin

Published in PLOS ONE (2012), 7(2), e31347

In this manuscript we report stable and transient means of plant mediated RNAi of *M. sexta* midgut genes.

Stable transgenic *N. attenuata* plants harboring a 312bp fragment of *Ms*CYP6B46 in an inverted repeat orientation (*ir*-CYP6B46) were generated to produce *Ms*CYP6B46 dsRNA. *M. sexta* larvae feeding such plants caused in decreased accumulation of CYP6B46 transcripts in larval midguts. The same 312bp cDNA was also cloned in an antisense orientation into a tobacco rattle virus vector and *Agro*-infiltrated into *N. attenuata* plants. Similar type of reduction in CYP6B46 transcripts were observed in midguts without reducing transcripts of the most closely related *Ms*CYP6B45. Both the technique provides a specific and robust means of silencing target gene in *M. sexta* larvae, however transient system is better suited for high throughput analyses. We also show longer the dsRNA of target gene higher is the efficiency of silencing. This method can be adapted for rapidly screening the loss of function of *M. sexta* midgut genes.

Experimental design and reagents/materials/analysis tools provided by Ian T. Baldwin. Experiments performed and data analyzed by Pavan Kumar and Sagar Pandit. Manuscript written by Pavan Kumar, Sagar Pandit and Ian T. Baldwin.

MANUSCRIPT II

A natural history driven, plant mediated RNAi based study reveals CYP6B46's role in a nicotine-mediated anti-predator herbivore defense

Pavan Kumar, Sagar S. Pandit, Anke Steppuhn and Ian T. Baldwin

Under review in PNAS

In this manuscript we used our *in house* developed plant mediated RNAi method to study how *M. sexta* larvae use hostplant defense-metabolite against native predatory spiders with the help of midgut expressed CYP6B46.

Transcriptome of larvae fed wild-type and nicotine-free *Nicotiana attenuata* plants, the larvae's native host, revealed that midgut expressed *Ms*CYP6B46 was strongly regulated by nicotine ingestion. By transforming *N. attenuata* to produce CYP6B46 dsRNA, planting them into native habitats and infesting them with larvae, we silenced larval *Ms*CYP6B46 expression and observed the behavior of larval predators. The attack behavior of a native wolf spider provided the key to understanding *Ms*CYP6B46's function: the spiders clearly preferred CYP6B46-silenced larvae, just as they had preferred larvae fed nicotine-deficient plants. Additional experiments demonstrated that *Ms*CYP6B46 allows a small amount (0.2%) of the ingested nicotine to move from midgut to hemolymph, providing larvae a means of exhaling nicotine into the headspace through their spiracles as a volatile anti-spider signal. Other abundant native predators which lack the spider's prey-assessment behavior (big eyed bugs and ant lion larvae) were insensitive to the larvae's ingested nicotine, suggesting that nicotine-externalization was specifically effective against spiders. This work demonstrates how plant chemical defenses, too toxic to be readily sequestered and largely excreted through the solid waste stream, can be re-purposed for defensive functions through the respiratory waste stream as a form of defensive halitosis.

Pavan Kumar, Sagar S. Pandit, Anke Steppuhn and Ian T. Baldwin designed and performed experiments, analyzed data and wrote the manuscript.

MANUSCRIPT III

Difference in nicotine metabolism of two herbivores of *Nicotiana attenuata* renders them differentially susceptible to a common native predator

Pavan Kumar, Preeti Rathi, Matthias Schoettner, Ian T. Baldwin and Sagar S. Pandit

Manuscript prepared for submission to Ecology letters

In this work we discovered the nicotine-coping strategies of various lepidopteran herbivores like *Manduca quinquemaculata, Spodoptera exigua, S. littoralis* and *Heliothis virescens* and compared the physiological and ecological advantages of nicotine-coping strategies of *S. exigua* with that of *M. sexta* larvae.

We tested if nicotine oxides are less toxic to *M. sexta* than nicotine and if larvae excrete them faster than nicotine, since we did not find them in our analysis and there were contradictroy reports in literature about their formation in *M. sexta* larvae. None of the oxides were found to be less toxic than nicotine and all were excreted at the same rate as that of nicotine. Next, we found that *S. exigua*, *S. littoralis* and *H. virescens* oxidize nicotine whereas *M. quinquemaculata* does not. Cotinine and cotinine-N-oxide (CNO) could be found in the hemolymph and frass, whereas nicotine N-oxide (NNO) was found only in the frass of nicotine oxidizing larvae. We used *S. exigua* as a nicotine oxidizing model to test if the nicotine oxides confer lower toxicity than nicotine to the larvae that synthesize these oxides; we fed these oxides to larvae through the diet and measured the larval mass and mortality. To these larvae nicotine was the most detrimental, whereas NNO was the least, suggesting that NNO is not only an oxidation product but it could also be a detoxification product. Further, we also studied the ecological benefits of nicotine-oxides to *M. sexta* and *S. exigua* by testing the deterrence ability of nicotine oxides against the nicotine-sensitive wolf spiders. None of the nicotine oxides deterred spiders. Only nicotine fed or coated larvae of both the species deterred spiders. We infer that oxidation of nicotine by *S. exigua, S. littoralis* and *H. virescens* could be due to a general spontaneous response to the ingested xenobiotic that most of the generalist herbivores show and all the oxides may not confer the detoxification effect. In fact, possession of nicotine oxides renders them susceptible to the spiders than the possession of unmetabolized nicotine. On the contrary, *M. sexta* is ecologically benefitted by keeping nicotine unmetabolized. Since *S. exigua* lowers its nicotine content by oxidation, it may provide a gateway for the nicotine-sensitive members of higher trophic levels in *N. attenuata*-hosted niche. Our results also suggest that host allelochemistry and selection pressure from

predators could have been two of many factors behind the specialization of *M. sexta* on nicotine containing plants.

Pavan Kumar, Sagar S. Pandit, Preeti Rathi and Matthias Schoettner designed and performed experiments, analyzed data and wrote the manuscript; Ian T. Baldwin wrote the manuscript.

Chapter 3

Manuscript I

Tobacco rattle virus vector: A rapid and transient means of silencing *Manduca sexta* genes by plant mediated RNA interference

Tobacco Rattle Virus Vector: A Rapid and Transient Means of Silencing *Manduca sexta* Genes by Plant Mediated RNA Interference

Pavan Kumar, Sagar Subhash Pandit, Ian T. Baldwin*

Department of Molecular Ecology, Max Planck Institute for Chemical Ecology, Jena, Germany

Abstract

Background: RNAi can be achieved in insect herbivores by feeding them host plants stably transformed to express double stranded RNA (dsRNA) of selected midgut-expressed genes. However, the development of stably transformed plants is a slow and laborious process and here we developed a rapid, reliable and transient method. We used viral vectors to produce dsRNA in the host plant *Nicotiana attenuata* to transiently silence midgut genes of the plant's lepidopteran specialist herbivore, *Manduca sexta*. To compare the efficacy of longer, undiced dsRNA for insect gene silencing, we silenced *N. attenuata*'s dicer genes (*Na*DCL1- 4) in all combinations in a plant stably transformed to express dsRNA targeting an insect gene.

Methodology/Principal Findings: Stable transgenic *N. attenuata* plants harboring a 312 bp fragment of *Ms*CYP6B46 in an inverted repeat orientation (*ir*-CYP6B46) were generated to produce CYP6B46 dsRNA. After consuming these plants, transcripts of CYP6B46 were significantly reduced in *M. sexta* larval midguts. The same 312 bp cDNA was cloned in an antisense orientation into a TRV vector and *Agro*-infiltrated into *N. attenuata* plants. When larvae ingested these plants, similar reductions in CYP6B46 transcripts were observed without reducing transcripts of the most closely related *Ms*CYP6B45. We used this transient method to rapidly silence the expression of two additional midgut-expressed *Ms*CYPs. CYP6B46 transcripts were further reduced in midguts, when the larvae fed on *ir*-CYP6B46 plants transiently silenced for two combinations of *Na*DCLs (DCL1/3/4 and DCL2/3/4) and contained higher concentrations of longer, undiced CYP6B46 dsRNA.

Conclusions: Both stable and transient expression of CYP6B46 dsRNA in host plants provides a specific and robust means of silencing this gene in *M. sexta* larvae, but the transient system is better suited for high throughput analyses. Transiently silencing *Na*DCLs in *ir*-CYP6B46 plants increased the silencing of *Ms*CYP6B46, suggested that insect's RNAi machinery is more efficient with longer lengths of ingested dsRNA.

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* E-mail: baldwin@ice.mpg.de

Introduction

RNA interference (RNAi), the double stranded RNA (dsRNA) mediated gene silencing was discovered in nematodes in 1998 [1]. During the RNAi process, dsRNA produced in the nucleus is transported to the cytoplasm; alternatively, exogenous dsRNA can be taken up by cells with the help of the cell surface protein, SID [from systemic RNAi deficient mutants (*sid*)] [2]. In the cytoplasm, dsRNA is cleaved by RNaseIII type enzymes (dicers) to produce approximately 22 bp fragments, called small interfering RNAs (siRNAs) [3,4]. One strand of the siRNA (guide strand) is incorporated into the RNA-induced silencing complex (RISC) with the perfectly complementary site in a target mRNA to form a guide strand-target mRNA duplex [5]. The target mRNA is then sliced by the Argonaute protein of RISC [6]. In plants and nematodes, RNAi is amplified by the activity of RNA-dependent RNA polymerases (RdRPs). This enzyme extends the guide strand that is bound to the target mRNA towards its 5′ end [3,7]. The long dsRNA formed in this process re-enters the RNAi cycle after it is cleaved by dicers. RNAi-mediated gene silencing becomes a systemic process as the siRNAs spread to neighboring cells to induce a fresh cycle of dicing and splicing Mlotshwa1 [3,7,8].

This endogenous gene silencing mechanism has been exploited as a reverse genetic tool for several model organisms and has also been proposed as a potentially useful tool for pest control [9,10]. However, there are two major limitations to the widespread use of RNAi in insects: 1) the generation of stably transformed lines of insects for the reverse genetics research is onerous, and 2) the apparent lack of key components of the RNAi pathway in insects, namely SIDs and RdRPs [11], which requires that large quantities of triggering siRNAs be continuously administered to sustain gene silencing. To understand if RNAi is functional in insects, researchers have delivered dsRNA to insects by various methods: feeding [12,13,14,15,16,17], injection [18] and exogenous application [19]. Interestingly, the success of silencing genes by these different delivery modes differs amongst the various insect orders:

Coleoptera [9,20], Diptera [21], Hemiptera [13,14], Hymenoptera [22], Isoptera [23], Lepidoptera [12,24] and Orthoptera [25]. As RdRP orthologs are thought to be absent in most orders of the insects [11], the spontaneous amplification of RNAi is considered unlikely and the silencing effects are thought to be transient. Hence, sustained RNAi would require a continuous input of large quantities of dsRNA [13,15,17]. This limitation of the RNAi procedures could be alleviated in the case of herbivorous insects if the insects' host plants could be transformed to express dsRNA targeting insect genes. This approach has been shown to be effective by several researchers [13,14,15,17]. Their work revealed that when insects feed on dsRNA-producing transgenic host plants, dsRNA molecules penetrate the herbivores' gut cells and reduce the expression of the target gene by post transcriptional gene silencing. This strategy, which recommended the use of stably transformed plants for achieving gene silencing in herbivorous insects was called plant mediated RNAi (in this paper abbreviated PMRi).

RNAi and PMRi in insects provide a new reverse genetics research tool which has the potential to enable 'real time' analysis of insect gene function during herbivorous insect-plant interactions, as well as a powerful new means of controlling pests. However, the process appears to be challenging to implement for one of the most specious orders of herbivorous insects, the Lepidoptera [24], and is constrained by the time and labor requirements of generating stably transformed plants. Given that microarray-based transcriptomics of insects feeding on plants have revealed a plethora of regulated genes in insect exomes, more high throughput means of PMRi are required. Pitino and colleagues suggested a transient transformation strategy against aphids based on the *Agro*-infiltration of *Nicotiana benthamiana* leaf discs, [14]; whereas, a plant-virus based RNAi technique with these qualities was suggested against nematodes [26,27]. Dubreuil *et al.* [26] and Valentine *et al.* [27] engineered the tobacco rattle virus (TRV) for dsRNA production in plants. These researchers transiently transformed *N. benthamiana* plants with viral-based constructs and demonstrated RNAi in nematodes feeding on these plants. Here we report the development of a similar plant-virus based dsRNA producing system (VDPS) for the silencing of lepidopteran genes.

To examine the utility of VDPS against insects, we attempted to silence genes of the herbivorous insect, *Manduca sexta* (Sphingidae, Lepidoptera), through its native host plant, *N. attenuata*. This plant-herbivore system was chosen because stable as well as TRV-based transient transformation systems are well established for *N. attenuata* [28,29] and the trophic delivery of dsRNA has recently been reported for *M. sexta* [16]. Secondly, unlike *Bombyx mori* or *Helicoverpa armigera* which represent domesticated and pest insect models, respectively, *M. sexta* is an ecological insect model whose interactions with its host are well characterized. Therefore, the development of a VDPS for *M. sexta* would be a useful tool for the study of ecological interactions. As the first gene targets for silencing, we selected three midgut expressed cytochrome P450 (CYP) genes. We compared the silencing efficiency of stable PMRi and the new transient VDPS, for one of the candidate CYPs. Second, Terenius *et al.* stated that, "it is always a concern that based on the mechanism of gene silencing, RNAi treatments may in some cases induce off-target effects" [24]; considering this possibility, we examined the specificity of VDPS for the silencing of *M. sexta* genes and analyzed "off-target" effects on the expression of CYPs that share the highest sequence identity with the three targeted CYPs. In addition, we silenced *N. attenuata*'s four dicer (DCL) genes during PMRi to evaluate if ingestion of longer undiced dsRNA increases the silencing efficiency of the targeted insect genes.

Results

Selection of *Ms*CYPs for silencing test

In preparation for a more detailed analysis of *M. sexta* larvae's remarkable ability to tolerate dietary nicotine, we found literature that reported the transcripts of three *Ms*CYPs (CYP6B46, CYP4M1 and CYP4M3) to be up-regulated in response to dietary nicotine intake [30,31].

To identify the CYPs that were most closely related to the three target candidates CYP6B46, CYP4M1 or CYP4M3, and hence potentially at the risk of being co-silenced by the RNAi procedure, we aligned all 23 *Ms*CYP nucleotide sequences that were available as complete coding sequences in the NCBI database to construct a phylogenetic tree (Fig. 1A). We found that CYP6B45 had the highest sequence similarity to CYP6B46 (80.2%), whereas CYP4M2 was similar to both CYP4M1 (63.5%) and CYP4M3 (55.2%). Notably, CYP4M1 and CYP4M3 shared 53.3% sequence identity with each other. Four homologous regions of >21nt were identified in the alignment of CYP6B46 and CYP6B45 that were exactly identical (+231 to +267, +415 to +437, +1306 to +1334 and +1384 to +1430). No homologous regions of >21nt identical bases could be identified in the alignments of CYP4M1, CYP4M2 and CYP4M3.

CYP6B46, CYP4M1 and CYP4M3 had ORFs of comparable lengths (1524, 1515 and 1503 bp, respectively). In order to keep the length of the undiced dsRNA precursor uniform for all three genes we cloned the cDNA fragments of ≥300 bp from each of these genes into the VDPS vector. To accomplish this, we analyzed these sequences for the availability of primer binding sites ≥300 bp apart from each other and selected regions to be cloned in each candidate cDNA. The similarity of this selected ≥300 bp region from CYP6B46 with its homolog in CYP6B45 was 80.4%. This region contained one >21nt (+415 to +437 of the ORF = +112 to +134 of selected ≥300 bp fragment) stretch that was identical in the two aligned fragments (Fig. S1A). The ≥300 bp regions selected from CYP4M1 and CYP4M3 were homologous to each other (54.1% similar), whereas their similarity with the homologous region from CYP4M2 was 64.5% and 57.1%, respectively (Fig. S1B). The exact sizes of these selected regions were 312 bp (+301 to +612), 338 bp (+1000 to +1337) and 322 bp (+966 to +1287) in CYP6B46, CYP4M1 and CYP4M3, respectively.

PMRi is thought to mainly target genes that are expressed in gut tissues [13,15,17]. Therefore, to ascertain whether the candidate genes (CYP6B46, CYP4M1 and CYP4M3) were gut expressed, we profiled their transcripts along with the transcripts of the allied co-target (CYP6B45 and CYP4M2) genes in hemolymph, Malpighian tubules, fat body, foregut, midgut and hindgut. All five genes were found to have relatively higher expression levels in the gut regions as compared to the other tissues ($p \leq 0.05$; Fig. 1B). The primers used for this profiling specifically amplified respective insect cDNA; they did not produce any amplicons when plant cDNA was used as a PCR template (Fig. S1C).

Stable transgenic plant mediated RNAi (PMRi) for *Ms*CYP6B46

We generated stable transgenic lines of *N. attenuata* plants transformed with a pSOL8 vector harboring an inverted repeat (*ir*) of the selected 312 bp CYP6B46 cDNA fragment (Fig. S2A). A single insertion of the transgene was confirmed by southern hybridization, in two T_2 generation lines (*ir*-CYP6B46 30-2 and *ir*-CYP6B46 416-3) that were generated from two independent transformation events (Fig. S2B). Transcription of 312 bp CYP6B46 'ir' insertions and the subsequent formation of diced

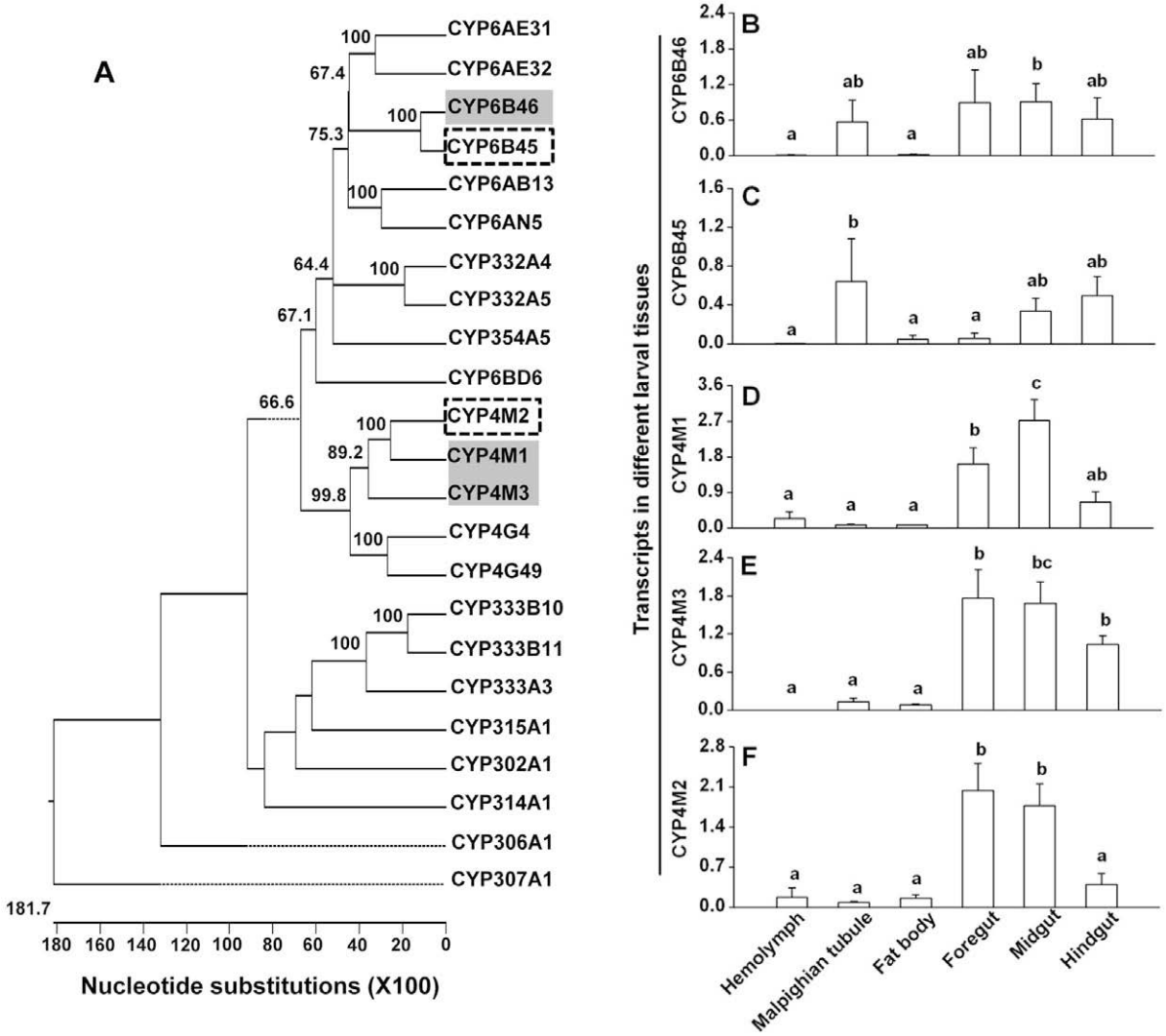

Figure 1. Selection of *M. sexta* CYPs for plant mediated RNAi (PMRi) and their spatial expression profiles. (**A**) Phylogenetic relationship among *M. sexta* CYPs (complete ORFs) as calculated by the Clustal-W program. CYPs selected for silencing by PMRi are shaded in gray and their most closely related CYPs that were analyzed for off-target co-silencing are in dashed boxes. Thousand bootstrapping trials were conducted (only the bootstrap values >50 displayed). Transcript levels (relative to ubiquitin) of (**B**) CYP6B46, (**C**) CYP6B45, (**D**) CYP4M1, (**E**) CYP4M3 and (**F**) CYP4M2 in hemolymph, Malpighian tubules, fat body, foregut, midgut and hindgut of 5th instar *M. sexta* larvae feeding on *N. attenuata* (WT) plants. Bars labeled with different letters indicate the significant differences as determined by one way ANOVAs ($p \leq 0.05$).
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small dsRNA (21–24nt) (smRNA) in the leaves of the *ir*-CYP6B46 lines were ascertained by northern hybridization (Fig. 2A). CYP6B46 smRNA was not detected in the leaves of controls wild type (WT) and empty pSOL8 vector (EV) plants. Morphology and development of the plants of both *ir*-CYP6B46 lines were similar to those of WT or EV plants.

Freshly hatched neonates of *M. sexta* larvae were placed on control and *ir*-CYP6B46 (30-2 and 416-3) lines and allowed to feed freely for 14 days. Using Northern hybridization, we confirmed the presence of CYP6B46 smRNA in their midgut (Fig. 2A). No CYP6B46 smRNA could be detected in the midguts of larvae that fed on the WT and EV plants (Fig. 2A). Further, CYP6B46 transcript levels in the midgut of these 14 d old larvae feeding on two *ir*-CYP lines were significantly reduced (three fold) compared to those of the larvae feeding on WT or EV plants ($p \leq 0.05$; Fig. 2B). Interestingly, the silencing of this gene had no effect on larval mass gain recorded after 14 d of feeding ($p > 0.05$; Fig. 2D).

PMRi mediated silencing was target gene specific

To ascertain if the silencing was specific to the target gene we quantified the transcript levels of the closely related off-target gene, *CYP6B45*. The transcript levels of *CYP6B45* in the midguts of larvae feeding on WT, EV and transgenic *ir*-CYP6B46 (30-2 and 416-3) plants did not differ ($p > 0.05$; Fig. 2C).

VDPS mediated silencing of *Ms*CYP6B46, *Ms*CYP4M1 and *Ms*CYP4M3

In order to rapidly and efficiently synthesize the dsRNA *in planta*, virus induced gene silencing (VIGS) vector pTV containing the antisense cDNA fragment (≥300 bp) of *Ms*CYP6B46, *Ms*CYP4M1 or *Ms*CYP4M3 (Fig. S3A) was *Agro*-infiltrated into *N. attenuata* WT leaves, as described by Saedler and Baldwin [29]. The smRNA produced in the leaves was detected by Northern hybridization, using the appropriate gene specific probe (Fig. 3A–

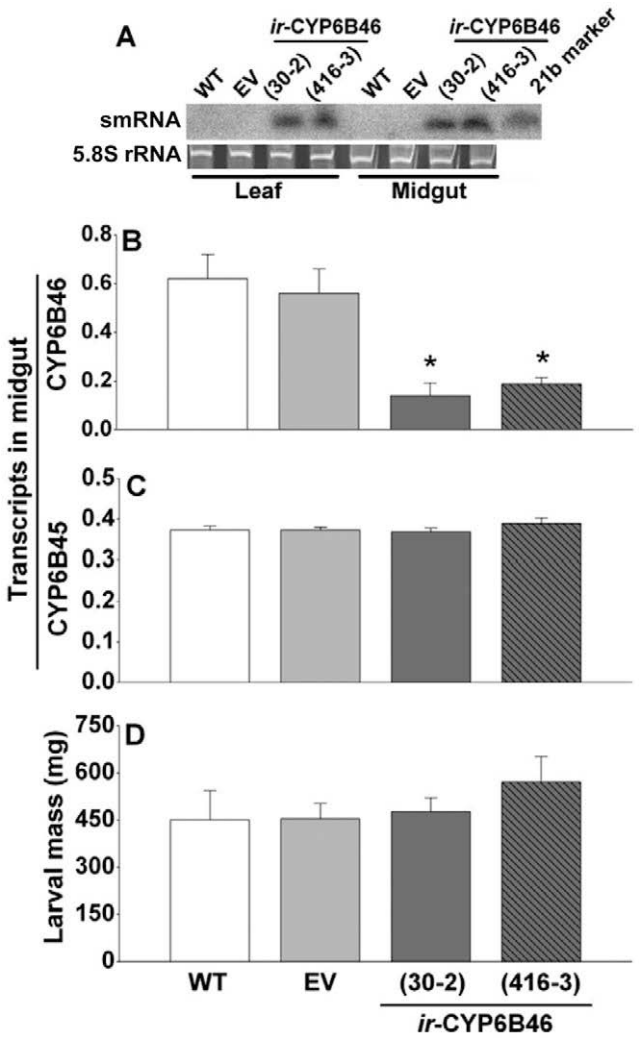

Figure 2. PMRi of *M. sexta* CYP6B46 using stably transformed *N. attenuata* plants. (**A**) Northern hybridizations revealed the presence of small RNAs of CYP6B46 in the leaves of two independent lines of stably transformed plants, *ir*-CYP6B46 (30-2) and *ir*-CYP6B46 (416-3), and in the midguts of 4th instar larvae feeding on these plants. RNA samples from leaves of WT and EV (empty vector transformed stable line) plants and from the midguts of larvae feeding on WT and EV leaves were used as negative controls. Similar fluorescence intensity of the ethidium bromide stained 5.8 S rRNA bands reflected the equal loading of LMW RNA. Low molecular weight RNA from leaf or midgut loaded on the gel in each lane was a pool of three biological replicates. smRNA length of 21 b denoted by marker. Transcript abundance (relative to ubiquitin) of: (**B**) CYP6B46 (target gene) and closely related (**C**) CYP6B45 (off-target) in the midguts of 4th instar larvae. (**D**) Larval mass of 4th instar larvae feeding on WT, EV, *ir*-CYP6B46 (30-2) and *ir*-CYP6B46 (416-3) *N. attenuata* plants for 14 days. Asterisk indicates the significant differences as determined by one way ANOVAs ($p \leq 0.05$).
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3C). For all three CYPs, the negative control leaves (EV) did not contain any of the target smRNAs.

Freshly hatched *M. sexta* neonates were transferred to VDPS-CYP6B46, -CYP4M1, -CYP4M3 and -EV plants. After 14 d of feeding, the midguts of these larvae were analyzed for the presence of the respective smRNAs by Northern hybridization. The expected smRNAs were detected in the midguts of larvae feeding on VDPS-CYP6B46, -CYP4M1 and -CYP4M3 lines (Fig. 3A–3C). The success of the silencing of the target gene was quantified by the reduction in the transcript levels of the target gene in the midguts of larvae feeding on the respective VDPS-CYP line, compared to larvae feeding on VDPS-EV. VPDS reduced CYP6B46 transcripts by three fold (Fig. 3D), reductions that were equivalent to that mediated by PMRi (Fig. 2B). Transcripts of CYP4M1 and CYP4M3 were reduced by 50% in the midguts of larvae feeding on the respective VDPS plants, as compared to that in the midguts of EV fed larvae ($p \leq 0.05$; Fig. 3F, 3H). Larval performance measured in terms of body mass after feeding for 14 d on VDPS-CYP6B46 and VDPS-EV plants was unchanged ($p > 0.05$; Fig. 3J): a result congruent with the larval mass gain observed on stable PMRi lines (*ir*-CYP6B46 and EV) (Fig. 2D). Larvae feeding on VDPS-CYP4M1 plants also did not show any difference in body mass as compared to that of larvae feeding on control EV plants ($p > 0.05$; Fig. 3J). However, the larvae feeding on VDPS-CYP4M3 plants gained significantly less mass compared to EV fed larvae ($p \leq 0.05$; Fig. 3J).

VDPS mediated silencing is target gene specific

Levels of CYP6B45 transcripts, the off-target gene, remained unchanged in the midguts of larvae feeding on EV or VDPS-CYP6B46 plants ($p > 0.05$; Fig. 3E), suggesting that the CYP6B46 silencing attained by VDPS was comparably specific to that attained by stable PMRi (Fig. 2C). Similarly, the silencing of CYP4M1 did not cause co-silencing of CYP4M3 and *vice versa* ($p > 0.05$) (Fig. 3F, 3H); moreover, larvae feeding on these two VDPS lines (-CYP4M1 and -CYP4M3) had similar CYP4M2 transcript levels in their midguts (compared to larvae feeding on EV; $p > 0.05$) (Fig. 3G, 3I).

Silencing of plant dicers enhances the silencing of CYP6B46 by PMRi

DCLs are involved in the biogenesis of smRNA by cleaving longer dsRNA. Four different types of DCLs are reported in higher plants. Their function has been found to overlap in plants, suggesting that one DCL can contribute to and/or compensate for the function of the others. Hence, more than one DCL might be involved in processing long dsRNA [32]. Efficiency of plant mediated silencing of *H. armigera* gene was previously shown to be increased by feeding long dsRNA that was obtained by silencing three Arabidopsis dicers 2, 3 and 4 [15]. We aimed to understand if the plant dicers functioned similarly in the *N. attenuata- M. sexta* model. To select the most effective combination of DCLs to silence, so as to increase the concentration of the retained longer dsRNA, we *Agro*-infiltrated all 16 combinations of the four *Na*DCL constructs into the stable PMRi line *ir*-CYP6B46 (30-2) (Fig. 4A). We found that silencing *Na*DCL 4, or the simultaneous silencing of any three *Na*DCLs significantly increased the accumulation of long dsRNA (102 bp that could be quantified by qRT-PCR) in plant leaves ($p \leq 0.05$; Fig. 4B, S4A). We selected the combinations of *Na*DCL 1, 3 and 4, and *Na*DCL 2, 3 and 4 for further experiments, based on the low variance as indicated by smaller standard errors of the long dsRNA transcript abundance mean values, as well as based on the previous PMRi report of the Arabidopsis *dcl* triple mutant [15]. Silencing of each *Na*DCL by the *Na*DCL 1, 3 and 4, or *Na*DCL 2, 3 and 4 VIGS construct combinations was confirmed by transcript quantification (Fig S4B-S4E). *M. sexta* larvae were fed *ir*-CYP6B46 (30-2) plants infiltrated with these *Na*DCL VIGS construct combinations. WT as well as *ir*-CYP6B46 (30-2) plants infiltrated with empty VIGS vector (EV) were used as controls. After feeding on the two combination *Na*DCL VIGS plants for 14 d, larval midguts showed 50% reductions in CYP6B46 transcripts as compared to those of larvae fed on *ir*-CYP6B46 (30-2)-EV plants ($p \leq 0.05$) (Fig. 4C), demon-

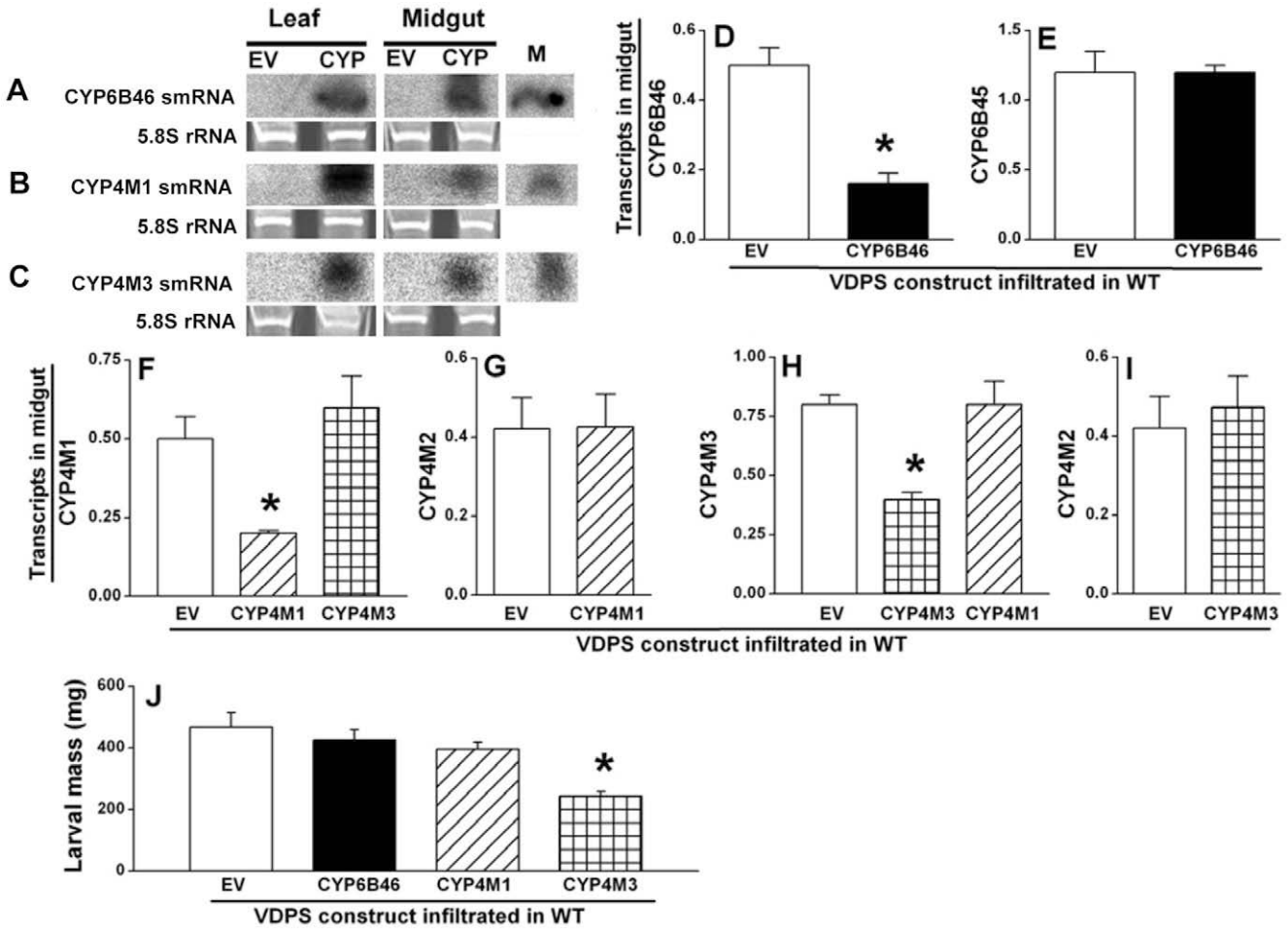

Figure 3. Efficiency and specificity of CYP6B46, CYP4M1 and CYP4M3 silencing by viral dsRNA-producing system (VDPS). Northern hybridization showing the smRNAs of (**A**) CYP6B46 (**B**) CYP4M1 and (**C**) CYP4M3 in WT leaves infiltrated with VDPS-EV, -CYP6B46, -CYP4M1 and -CYP4M3 constructs, respectively, as well as in the midguts of 4th instar larvae feeding on the leaves of plants inoculated with the respective constructs. Lane M shows 21 b oligonucleotide that was used as size marker as well as a positive control for hybridization. Similar fluorescence intensity of the ethidium bromide stained 5.8 S rRNA bands reflected the equal loading of LMW RNA. Transcript abundance (relative to ubiquitin) of the target genes. (**D**) CYP6B46 in the midguts of 4th instar larvae feeding on VDPS-EV and -CYP6B46, (**F**) CYP4M1 in the midguts of 4th instar larvae feeding on VDPS-EV, -CYP4M1 and -CYP4M3, and (**H**) CYP4M3 in the midguts of 4th instar larvae feeding on VDPS-EV, -CYP4M3 and -CYP4M1 plants, respectively. Transcript abundance (relative to ubiquitin) of closely related, off-target genes (**E**) CYP6B45 in the midguts of 4th instar larvae feeding on VDPS-EV and -CYP6B46, (**G**) CYP4M2 in the midguts of 4th instar larvae feeding on VDPS-EV and -CYP4M1, and (**I**) CYP4M2 in the midguts of 4th instar larvae feeding on VDPS-EV and -CYP4M3 plants, respectively. (**J**) Mass of 4th instar *M. sexta* larvae fed for 14 days on VDPS-EV, -CYP6B46, -CYP4M1 and -CYP4M3 plants. Asterisk indicates the significant differences as determined by one way ANOVAs ($p \leq 0.05$).
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strating that the silencing the plant's dicer machinery had increased the silencing efficiency of the PMRi by a factor of 2.

Discussion

PMRi has the potential of becoming a crop protection tool targeting insect pests with far greater specificity than currently available pesticides or xenobiotics such as the *Bt* toxin [15]. Especially in the case of lepidopteran insects, CYPs that detoxify plant defense compounds or synthetic insecticides are potential targets of RNAi. Silencing these CYPs, suppresses transcript levels of the targeted genes, attenuating their function, and influencing larval growth or survival [15,17,33]. Since they belong to multigene families, CYPs have also been proposed to be the ideal targets for combinatorial RNAi [17]. Therefore in this first attempt of insect gene silencing by transient plant viral system, we considered CYPs as valuable candidate genes.

Snyder and colleagues demonstrated that CYP4M1 and CYP4M3 were upregulated in *M. sexta* larval midgut in response to nicotine ingestion [31]. Similarly in a microarray analysis, Govind and colleagues showed that CYP6B46 was downregulated in the larval midgut, when fed on nicotine suppressed *ir*-PMT plants [30]. Based on their response to dietary nicotine, we selected these three genes (CYP6B46, CYP4M1 and CYP4M3) for the PMRi trial. *M. sexta* is the specialist herbivore of host plants that produce high concentrations of nicotine and is famous for having the highest tolerance to nicotine of any organism [34].

Several researchers demonstrated a successful insect gene silencing with the *Agrobacterium*-transformed stable or transient transgenic plant mediated RNAi [13,14,15,17]. We report similar silencing for the midgut-based CYP6B46 of *M. sexta*, suggesting that the PMRi is a reproducible and robust technique. Additionally, we showed that the silencing was highly specific and did not spread even to the most similar genes of the same

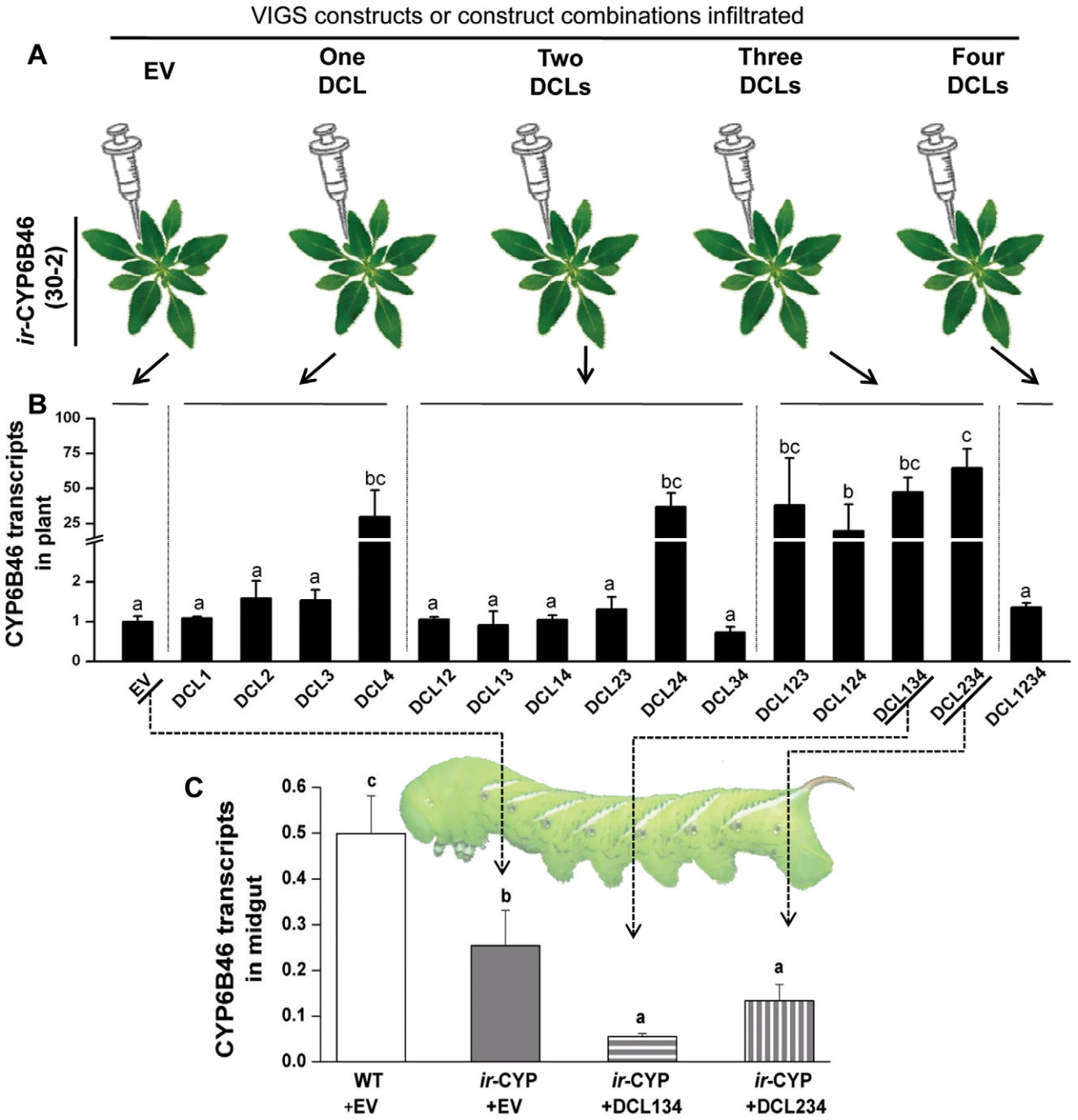

Figure 4. PMRi efficiency is increased after silencing *N. attenuata*'s dicer-like (DCL) genes. (**A**) Schematic representation of the silencing of *N. attenuata*'s four DCLs by virus induced gene silencing (VIGS), in *ir*-CYP6B46 (30-2) stably-transformed plants. *ir*-CYP (30-2) plants were *Agro*-infiltrated with pTVDCL harboring cultures (individually, and in all combinations of DCL1, DCL2, DCL3 and DCL4); pTV (EV) was used as a control. (**B**) Abundance, relative to *Na*Actin, of a 102 bp region of the 5′ end of the 312 bp *Ms*CYP6B46 fragment that the *ir*-CYP (30-2) *N. attenuata* plants were harboring in their genome. The plants had been previously *Agro*-infiltrated with EV or all combinations of vectors designed to silence the expression of the four *Na*DCLs. (**C**) Transcript abundance of CYP6B46 (relative to ubiquitin) in the midguts of 4th instar *M. sexta* larvae, when fed *N. attenuata* leaves containing no *Ms*CYP6B46 dsRNA (WT+EV), small (diced) *Ms*CYP6B46 dsRNA (*ir*-CYP6B46+EV) and on leaves of plants containing higher concentration of longer (102 bp detected by qPCR) *Ms*CYP6B46 dsRNA fragments (*ir*-CYP6B46+ DCL134 and *ir*-CYP6B46+ DCL234). See Fig. S4A and Table S1 for the design of the primers used in the transcript quantification. Bars labeled with different letters indicate significant differences as determined by one way ANOVAs ($p \leq 0.05$).
doi:10.1371/journal.pone.0031347.g004

family. Such specificity and reproducibility was important to demonstrate, as the RNAi in Lepidoptera has recently been suggested to be dependent on the insect, gene, gene function, organ of expression and mode of delivery [2,24]. More importantly for our aims, this work demonstrates that the *M. sexta*- *N. attenuata* ecological model was amenable to PMRi.

Successful PMRi in *M. sexta* allowed us to use it as benchmark for developing an easier and faster VDPS method. While trying the VDPS that was previously successful against root nematodes [26,27], we found that the silencing efficiency of VDPS and PMRi was similar. The VDPS experiments could be accomplished within three months in contrast to the PMRi procedure that requires a year of laborious screening of plants through two generations. Since VDPS is a more rapid technique, we could screen three CYPs in a short time and found CYP4M3 to be good candidate for further research. Since the larval growth was reduced while feeding on nicotine containing VDPS-CYP4M3 plants (Fig. 3J), this gene among the three CYPs tested, may play a central role in increasing *M. sexta*'s tolerance to nicotine ingestion.

Similar to stable PMRi, VDPS was also found to be highly specific in its silencing of all three candidate genes. PMRi is clearly the method of choice for crop protection in countries which allow the growth of transgenic crops and could be of immediate utility in the control of polyphagous pests such as *H. armigera*. However, VDPS could be a method of choice for high throughput reverse genetic screens of potential genetic targets in insect pests, as well as enabling research into a multitude of unanswered ecological questions at the molecular level.

The length of dsRNA is often of concern in RNAi experiments. In most of the experiments on insects that were based on the trophic delivery of dsRNA, different lengths of dsRNA (300–520 bp) has been used [2]. In the TRV based VDPS used against nematodes, inserts greater than 150 bp were recommended [27]. However, cloning of small sense or antisense fragments and especially small hairpins were also shown to be effective in the TRV vectors [27,35]. Therefore the standardization of insert length would be an important consideration for the future VDPS experiments. We addressed the issue of dsRNA length in the PMRi experiment. Silencing of CYP6B46 was enhanced after increasing the length of ingested dsRNA by silencing the plant's dicers. These results were consistent with the findings in *H. armigera* [15] and are consistent with the hypothesis that the efficiency of RNAi depends on the length of the ingested dsRNA. Recent discoveries have shown that the dsRNase from *B. mori* midgut is synthesized in midgut cells and subsequently secreted into the lumen [36,37,38]. Thus, it is possible that the lepidopteran dicers that function in extremely alkaline environments of the midgut are specialized and possess different dicing properties than do the plant dicers; consequently, insect-dicer diced smRNA might be more effective than the plant-dicer diced smRNA in gene silencing in insects.

We conclude that stable PMRi can be a specific and robust system of gene silencing in *M. sexta*. While retaining all the virtues of PMRi, VDPS promises to be a rapid and high throughput alternative, suitable for ecological research. Silencing of plant dicers in PMRi lines revealed that similar to the results obtained in *H. armigera*, the gene silencing effect is enhanced in *M. sexta* suggesting that plant and insect RNAi machinery respond differently to the varying lengths of dsRNA.

Materials and Methods

Plant material

N. attenuata 30× inbred seeds, which were originally collected in 1988 from a native population at Utah (United States) were used for the generation of *Agrobacterium tumefaciens* mediated stable transgenic as well as VDPS lines in all the experiments. The seeds were germinated on sterile Gamborg B5 medium (Sigma, Germany) after 1 h of treatment with 50× (V/V) diluted smoke (House of Herbs) and 1 μM GA_3. Ten days after germination, seedlings were transferred to Teku pots containing peat-based substrate, and after an additional 10 to 12 d, the plantlets were transplanted into individual 1L pots with the same substrate. In the glasshouse, plants were grown at 24°C to 26°C, relative humidity approximately 55%, and supplemented with light from 400- and 600-W sodium lamps (Philips) for 16 h [39].

Insect culture

Eggs from an in-house *M. sexta* colony were stored in a growth chamber (Snijders Scientific) at 26°C- 16 h light, 24°C- 8 h darkness and 65% relative humidity, until the larvae hatched. Three freshly hatched neonates were placed on each rosette-stage *N. attenuata* plant. Fourteen day old fourth instar larvae were used in all assays, unless specified otherwise.

Analysis of *Ms*CYP sequences and their selection for cloning in PMRi or VDPS vector

Sequences of 23 *M. sexta* CYPs were retrieved as complete coding sequences from NCBI. Their ORFs were aligned and a phylogenetic tree was constructed using Clustal W, with 1000 bootstrapping trials, to identify the most closely related *Ms*CYPs to the preselected targets of gene silencing: CYP6B46 (GU731529), CYP4M1 (GU731525) and CYP4M3 (GU731527).

Sequences of candidate gene cDNA fragments (≥300 bp) to be cloned in the PMRi or VDPS vector were selected based on two criteria: 1) the availability of primer binding sites ≥300 bp apart from each other in the candidate cDNA, according to the 'Primer3' online utility [40] and 2) the degree of similarity of this ≥300 bp fragment with their homologous region in the allied genes. This was considered in order to avoid co-silencing of closely related genes due to their high sequence similarity and to enable detection of 'off-target' effects of gene silencing [24]. Thus, CYP6B46 fragment was aligned with its homolog from CYP6B45 (GU731528), and the CYP4M1 and CYP4M3 fragments (which are already highly homologous to each other) were aligned with their closest homolog, CYP4M2 (L38671) (Fig. S1A, S1B).

Larval tissues

Hemolymph, Malpighian tubules, fat body, foregut, midgut and hindgut were collected from the fifth instar larvae for transcript profiling of the selected *Ms*CYPs. Hemolymph of each larva was collected separately by clipping the horn. Then, these larvae were dissected in 0.15 M NaCl under a dissecting microscope. The 14 d old fourth instar larvae used in all gene silencing assays were also dissected in 0.15 M NaCl to isolate their midguts. Dissected gut tissues were carefully washed in 0.15 M NaCl to remove any adhering plant material. All tissues were stored in TRI regent (Invitrogen) as recommended by the manufacturer, until further use.

Total RNA isolation

Total RNA was extracted from TRI regent stored tissues using manufacturer's protocol. Isolated total RNA was always subjected to TURBO DNase (Ambion) treatment, according to the manufacturer's protocol.

Real time quantitative PCR

All the primer sequences used for real time quantitative PCR (qRT-PCR) are listed in Table S1. These primers were designed from the transcript sequences retrieved from NCBI, using Primer3 [40], to amplify ≥100 bp in the respective template cDNA, upstream of the selected ≥300 bp regions. This upstream position was targeted because, in the organisms such as plants, nematodes and fruit flies in which the RNAi pathway has been well

characterized, silencing is known to spread in a 3′ to 5′ direction of the targeted mRNA [3]; hence the 5′ region of the target mRNA is longer lived and more available for qRT-PCR based quantification than regions in the 3′ region. For CYP6B46, CYP6B45, CYP4M1, CYP4M2 and CYPM3, the size of this amplicon was 118BP (+144 to +261), 105 bp (+613 to +718), 101 bp (+868 to +968), 166 bp (+453 to +619) and 130 bp (+703 to +832), respectively. The specificity of these primers for insect cDNA was confirmed by a PCR with *N. attenuata* (WT) leaf cDNA.

For each sample, 500 ng of total RNA was used for cDNA preparation using oligo(dT)$_{18}$ primer and SuperScript II enzyme (Invitrogen) with the manufacturer's recommendations. All the qRT-PCRs were performed with a Mx3005P Multiplex qPCR system (Stratagene) and the qPCR core kit for SYBR Green I (Eurogentec). Relative quantification of mRNA levels was performed by the comparative Δ cycle threshold (CT) method using the *Ms*Ubiquitin mRNA as an internal standard. All the qPCRs were performed using the following conditions: initial denaturation step of 95°C for 30 s, followed by 40 cycles each of 95°C for 30 s and 60°C for 1 min, with a final extension step of 95°C for 30 s and 60°C for 1 min. All the results were obtained from at least five independent biological replicates and two technical replicates.

Plant transformation

Stable transgenic inverted repeat-*Ms*CYP6B46 (*ir*-CYP6B46) *N. attenuata* lines were generated by transforming the recombinant pSOL8 transformation vector [28]. Vector contained 300 bp fragment of *M. sexta* CYP6B46 gene in an inverted repeat orientation along with the hygromycin phosphotransferase (*hptII*) gene providing hygromycin resistance as a selectable marker (Fig. S2A) [41]. Screening of the transgenic lines followed the protocol recommended by Gase *et al.* [42]. Two homozygous independently transformed *ir*-CYP6B46 lines (30-2 and 416-3) harboring single insertions of the *hptII* marker gene were used for further studies. Wild type (WT) and the stable transgenic empty pSOL8 vector (EV) containing [43] plants were used as negative controls.

Southern Hybridization

A modified cetyltrimethylammonium bromide method described by Rogers and Bendich [44] was followed for genomic DNA extraction from the fully expanded rosette leaves of WT, *ir*-CYP6B46 (30-2) and *ir*-CYP6B46 (416-3) *N. attenuata* plants. For Southern-blot hybridizations 10 µg of the genomic DNA was completely digested with *HindIII* and *EcoRV*, separately. It was then size fractionated on 1% (w/v) agarose gel, and blotted onto a nylon membrane (GeneScreenPlus; Perkin-Elmer) by capillary transfer. Hybridization and detection of the insertion number was performed as described by Gase *et al.* [42].

Virus induced plant gene silencing (VIGS)

A VIGS system based on the TRV was used to silence *N. attenuata* DCLs, as described by Saedler and Baldwin [29]. VIGS vector (pTV) harboring ≥300 bp fragments of *Na*DCL1 (JN032013), *Na*-DCL2 (JN032015), *Na*DCL3 (JN032015), and *Na*DCL4 (JN032016) (Bozorov *et al.*, in review) were used for VIGS. A vector without insert (EV) was used as a negative control. To silence the four *Na*DCLs in various combinations, the *Agrobacterium* cultures containing respective DCL-VIGS constructs were mixed in equal proportions before infiltration in three *ir*-CYP6B46 (30-2) plants. Primers were designed to amplify 102 bp of the cloned and expressed *Ms*CYP6B46 dsRNA in leaf (Fig. S4A; Table S1). Plants with DCL-VIGS combination retaining higher levels of 102 bp *Ms*CYP6B46 transcripts were detected by qRT-PCR and were selected for feeding *M. sexta* larvae. Silencing of each *Na*DCL used in the selected VIGS combinations was confirmed by the qRT-PCR based transcript quantification (Fig S4B- S4E). Prior to this work, it was confirmed that the primers used for *Na*DCL transcript quantification (Table S1) produced a single amplicon with *N. attenuata* (WT) cDNA (Fig S4F).

Plant tissue, RNA isolation, cDNA synthesis and qRT-PCR

Fully expanded *N. attenuata* rosette leaves were used in the entire analysis. They were harvested and immediately frozen in liquid nitrogen. The protocols used for insect tissues were also followed for their RNA isolation, cDNA synthesis and qRT-PCR. *Na*Actin was used as an internal standard for qRT-PCR. All the results were obtained from three independent biological replicates and two technical replicates.

Plant-Virus based dsRNA Producing System (VDPS)

N. attenuata VIGS system was modified with the substitution of inserted plant cDNA fragment by the insect cDNA fragment. Selected ≥300 bp fragment of *Ms*CYP6B46 or *Ms*CYP4M1 or *Ms*CYP4M3 was cloned in the pTV vector in an antisense orientation (Fig. S3) and *Agro*-infiltrated into WT plants to express ≥300 bp dsRNA of the respective cloned fragment. An empty pTV vector (EV) was used as a negative control. These transformed plants were fed to *M. sexta* larvae to silence the expression of *Ms*CYP6B46, *Ms*CYP4M1 and *Ms*CYP4M3, respectively.

Low Molecular Weight (LMW) RNA isolation

For the isolation of low molecular weight (LMW) RNA, the total RNA (from insect midgut as well as from leaves) was subjected to PEG (10%) precipitation in presence of 1 M NaCl. Subsequently, it was precipitated using isopropyl alcohol (0.8 volumes) by incubating overnight at −20°C.

Northern blotting and hybridization for the detection of smRNA

Fifty µg LMW RNA (pool of three biological replicates from insect midguts or leaves) was separated under denaturing conditions (8 M urea) in 15% acrylamide gel, stained with ethidium bromide and visualized with the help of a UV transilluminator. Similar fluorescence intensity of the 5.8 S rRNA bands reflected the equal loading of LMW RNA. The RNA was transferred to a nylon membrane (GeneScreenPlus; Perkin-Elmer) by electro-blotting at 400 mA for 1 h, using 0.5× TBE as a transfer buffer. Fragments of *Ms*CYP6B46 were PCR amplified using primer pair #4 (Table S1) for the preparation of the probe. Ten ng of these amplicons were labeled with α-^{32}P using the Rediprime II DNA labeling system (Amersham Biosciences). Hybridization and screening of the blot performed as previously explained, except that the blots were exposed to the Fujifilm (www.fujifilm.com) BAS-MS imaging plates for 10 to 12 days until no further increase in signal was observed [45].

Statistical analysis

All statistical analyses were performed with StatView version 5 (SAS Institute Inc.). Significance of variance was determined after the one way ANOVA ($P>0.05$) and was represented in all the graphs as ±S.E.

Supporting Information

Figure S1 Alignments of *Ms*CYPs cDNA regions selected for cloning; insect cDNA specific amplification by qRT-

PCR primers. Sequence alignments of *M. sexta* CYP cDNA fragments that were selected for PMRi experiments, with the homologous sequences of respective allied genes that were tested for off-target co-silencing effects (see Fig. 1A) (**A**) Alignment of the selected CYP6B46 cDNA fragment with the homologous fragment from the CYP6B45 cDNA. (**B**) Alignment of selected cDNA fragments of CYP4M1 and CYP4M3 with each other and with the homologous cDNA fragment from CYP4M2. (**C**) RT-PCR analysis showing that the primers used for the SYBR-Green qRT-PCR produced single amplicons (resolved on 2% agarose gel) with *M. sexta* cDNA and did not produce amplicons with *N. attenuata* leaf cDNA, demonstrating that the primers were insect cDNA specific. A 100 bp ladder was used as a size marker.
(TIF)

Figure S2 Map of plant stable transformation vector and detection of a single transgene insertion in the transformed lines. (**A**) A map of pSOL8 vector used for *Agrobacterium tumefaciens* mediated plant transformation harboring the inverted repeat (separated by the *pdk i3* intron) of a 312 bp fragment of *M. sexta*'s CYP6B46 cDNA. (**B**) Southern hybridization after *HindIII* digested genomic DNA, showing the presence of a single insertion of transgene in both the independently transformed (30-2 and 416-3) *N. attenuata* lines; WT control shows absence of transgene insertion. 1 kb DNA ladder was used as a size marker.
(TIF)

Figure S3 Map of viral dsRNA-producing system (VDPS) vector. A map of tobacco rattle virus (TRV) based plant (transient) transformation vector pTV. A ≥300 bp stretch from *M. sexta*'s CYP6B46/CYP4M1/CYP4M3 cDNA was cloned in an antisense orientation into pTV for VDPS. *N. attenuata* (WT) plants were transiently transformed with this recombinant vector by *Agro*-infiltration and *M. sexta* larvae were fed on the leaves of inoculated plants to silence the expression of CYP6B46/CYP4M1/CYP4M3 genes, respectively, in their midguts.
(TIF)

Figure S4 Schematic representation of cDNA regions used for *Ms*CYP6B46 transcript profiling in leaves and larval midguts. For transcript quantification in plant material, a 102 bp fragment residing inside the 312 bp region (300 to 612 b of the ORF) that was cloned into the pSOL8 vector in an inverted repeat orientation was used. After silencing plant's four DCL genes, the abundance of the transcripts of this fragment increased. Since this fragment was longer than the diced transcripts (21–24 bp), it provided a measure of the abundance of undiced transcripts. For the quantification of transcripts in larval midguts, another100 bp fragment, located 5′ of the cloned region was used. Using a fragment outside the cloned region ensured that the quantification of endogenous gene silencing would not be confounded by undiced dsRNA or vector-born transcripts. Transcript abundance of *N. attenuata*'s (**B**) DCL1, (**C**) DCL2, (**D**) DCL3 and (**E**) DCL4 in the leaves of WT *Agro*-infiltrated with EV, *ir*-CYP6B46 (30-2) *Agro*-infiltrated with EV or DCL1, 3 and 4 or DCL2, 3 and 4, respectively. (**F**) RT-PCR analysis showing that the *Na*DCL and *Na*Actin primers used for the qRT-PCR, produced single amplicons (resolved on 2% agarose gel) with *N. attenuata* (WT) cDNA. A 100 bp ladder was used as a size marker. Bars labeled with different letters indicate significant differences as determined by one way ANOVAs ($p \leq 0.05$).
(TIF)

Table S1 *M. sexta* and *N. attenuata* gene primers.
(DOC)

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Author Contributions

Conceived and designed the experiments: ITB. Performed the experiments: PK SSP. Analyzed the data: SSP PK. Contributed reagents/materials/analysis tools: ITB. Wrote the paper: ITB SSP PK.

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Supporting information

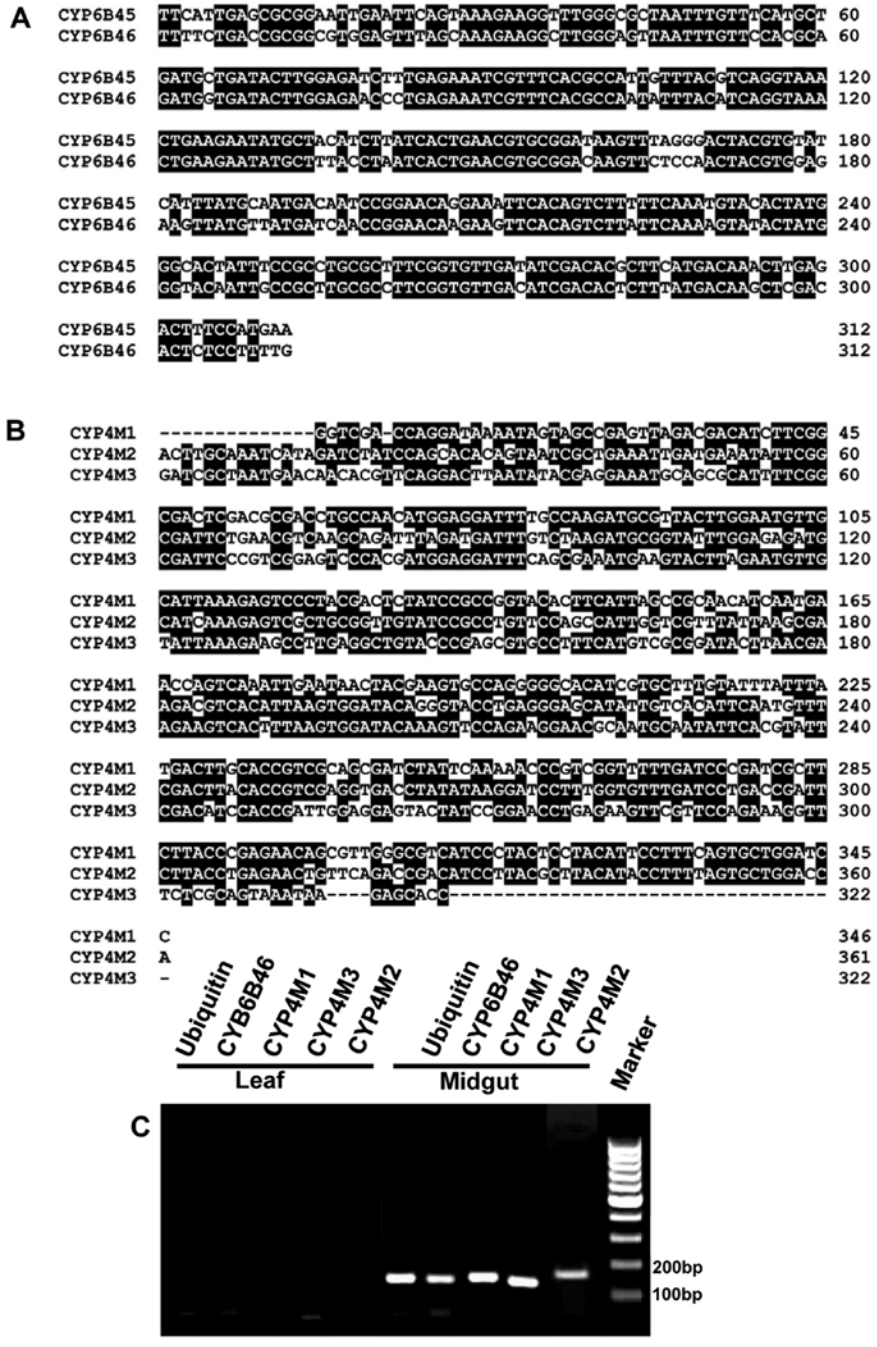

Figure S1 Alignments of *Ms*CYPs cDNA regions selected for cloning; insect cDNA specific amplification by qRT PCR primers.

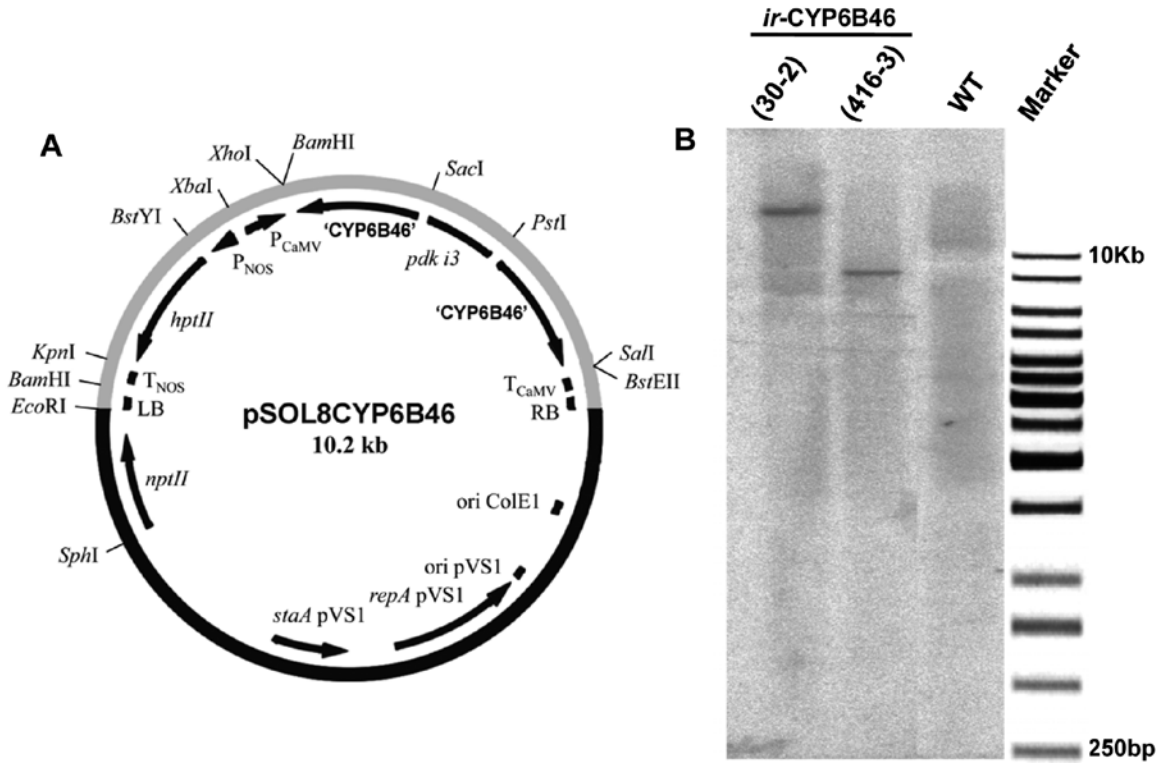

Figure S2 Map of plant stable transformation vector and detection of a single transgene insertion in the transformed lines.

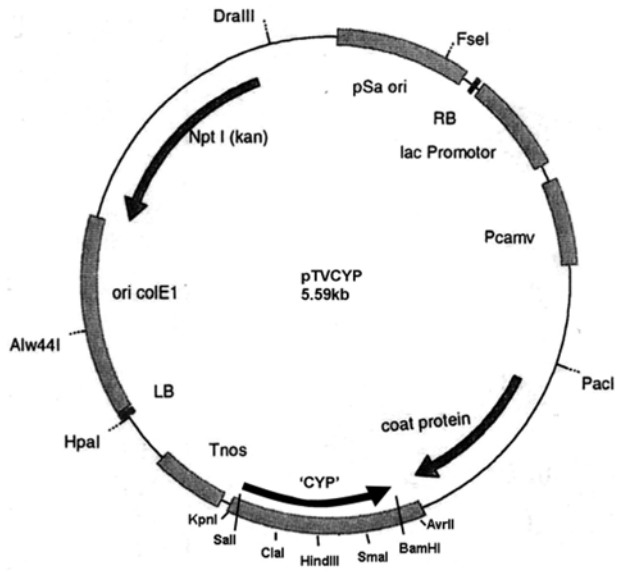

Figure S3 Map of viral dsRNA-producing system (VDPS) vector.

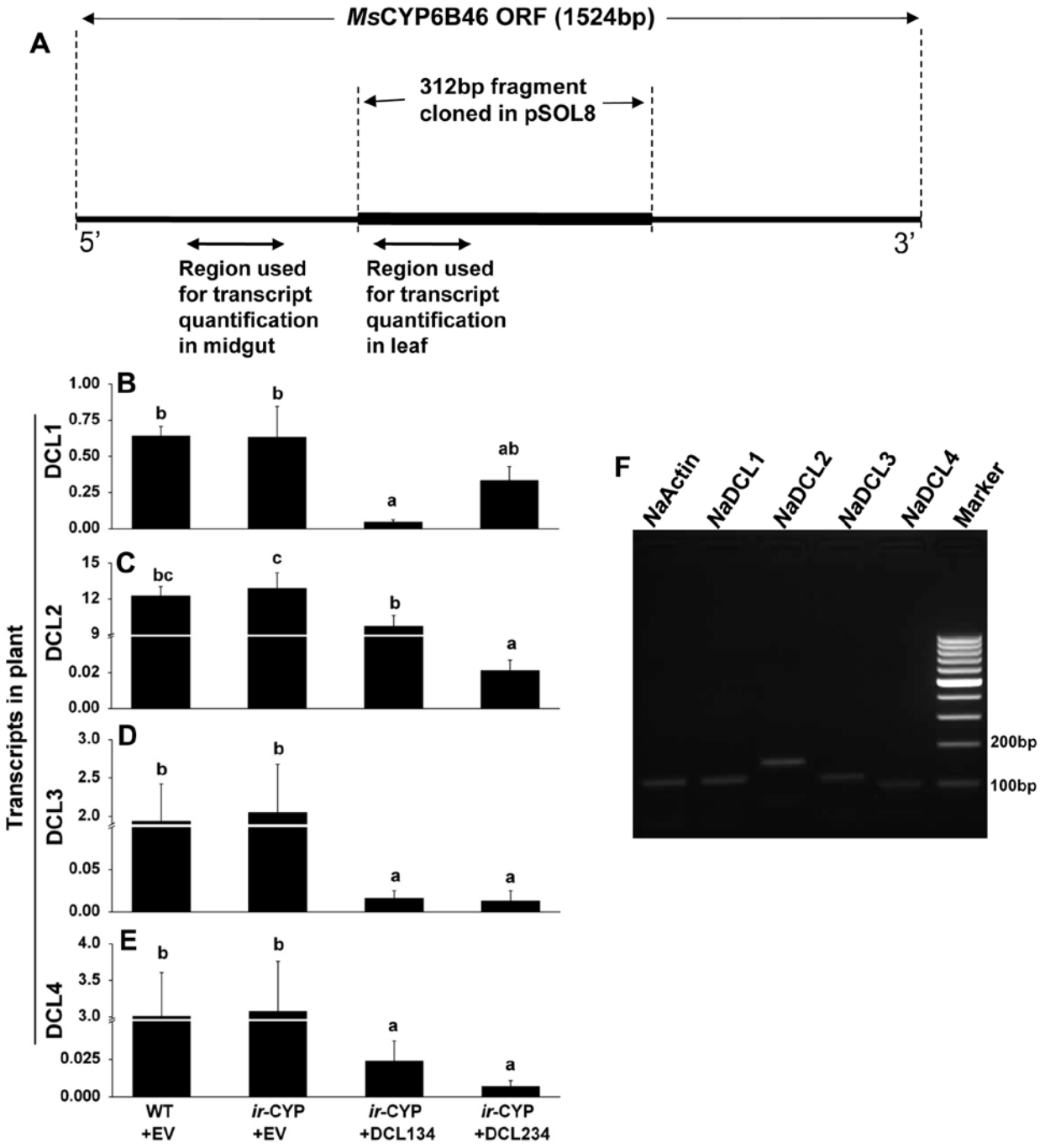

Figure S4 Schematic representation of cDNA regions used for *Ms*CYP6B46 transcript profiling in leaves and larval midguts.

Table S1. *M. sexta* and *N. attenuata* gene primers

Primer pair no.	Gene	Primer sequences (5'-3')	Use
1	***MsUbiquitin***	**For-** AAAGCCAAGATTCAAGATAAG **Rev-** TTGTAGTCGGATAGCGTGCG	Internal control for *M. sexta* transcript quantification
2	***MsCYP6B46***	**For-** GTGCCTATTACTCCGCGATCTA**Rev-** CCAAGCCTTCTTTGCTAAACTCC	Transcript quantification and silencing efficiency testing of *M. sexta* CYP6B46
3	***MsCYP6B46***	**For-** GCAGATGGTGATACTTGGAGAA **Rev-** GTCCGCACGTTCAGTGATTAG	Transcript quantification of CYP6B46 dsRNA in DCL/s silenced leaf
4	***MsCYP6B46***	**For-** TTTTCTGACCGCGGCGTGG **Rev-** AAAAGGAGAGTGTCGAGCTTG	Radiolabelled probe making for detection of *Ms*CYP6B46 small RNA
5	***MsCYP4M1***	**For-** GCTGAAAGAGATGGGGAAATC **Rev-** CAAAACGTCAACCCAGAAGC	Transcript quantification and silencing efficiency testing of *M. sexta* CYP4M1
6	***MsCYP4M1***	**For-** CAGGATAAAATAGTAGCCGAG **Rev-** CAGCACTGAAGGGAATGTAG	Radiolabelled probe making for detection of *Ms*CYP4M1 small RNA
7	***MsCYP4M2***	**For-** GGTGCAGAATGTCGGCAAATC **Rev-** TCTTCCGAGTGCGCAGATAG	Transcript quantification and co-silencing efficiency testing of *M. sexta* CYP4M2
8	***MsCYP4M3***	**For-** AGACGTGCAGTCAAAGACCTG **Rev-** CCATCCGACTTTTCTTACCG	Transcript quantification and silencing efficiency testing of *M. sexta* CYP4M3
9	***MsCYP4M3***	**For-** GATCGCTAATGAACAACACGT **Rev-** GGTGCTCTTATTTTCTGCGA	Radiolabelled probe making for detection of *Ms*CYP4M3 small RNA
10	***MsCYP6B45***	**For-** GAAATGGATAAATTGGTTTTGACC **Rev-** TTATTTTGACAGAGAAGATTGAGG	Transcript quantification and co-silencing efficiency testing of *M. sexta* CYP6B45

11	***NaActin***	**For-** GGTCGTACCACCGGTATTGT **Rev-** GTCAAGACGGAGAATGGCAT	Internal control for *N. attenuata* transcript quantification
12	***HptII***	**For-** CGTCTGTCGAGAAGTTTCTG **Rev-** CCGGATCGGACGATTGCG	Radiolabelled probe making for detection of transgene inserts in *N. attenuata*
13	***NaDCL1***	**For-** CCAGGCACAGGGAATTTTATC **Rev-** AGGTGAACCAACTTTGAGCTG	Silencing efficiency testing of *N. attenuata* DCL1
14	***NaDCL2***	**For-** CGATGAAGAATTGCTGATGC **Rev-** GCCTGAGCTGAGAAAGGCAC	Silencing efficiency testing of *N. attenuata* DCL2
15	***NaDCL3***	**For-** GCACTGATGGTGACATCTGC **Rev-** CACTGCTAGGTTGAGCTTTGG	Silencing efficiency testing of *N. attenuata* DCL3
16	***NaDCL4***	**For-** ACCAAGTGCTGCAACTTCAC **Rev-** TCCTTCTCTGGTTTCTGAACTG	Silencing efficiency testing of *N. attenuata* DCL4

Chapter 4

Manuscript II

A natural history driven, plant mediated RNAi based study reveals CYP6B46's role in a nicotine-mediated anti-predator herbivore defense

A natural history driven, plant mediated RNAi based study reveals CYP6B46's role in a nicotine-mediated anti-predator herbivore defense

Pavan Kumar[1], Sagar S. Pandit[1], Anke Steppuhn[a] , and Ian T. Baldwin[2]

Max-Planck-Institute for Chemical Ecology, 07745 Jena, Germany

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Manduca sexta larvae are the most nicotine-tolerant organisms known, but the molecular mechanisms of their exceptional nicotine tolerance remain unknown. Querying the transcriptome of larvae fed wild-type and nicotine-free Nicotiana attenuata plants, the larvae's native host, revealed that midgut expressed MsCYP6B46 was strongly regulated by nicotine ingestion. By transforming N. attenuata to produce CYP6B46 dsRNA, planting them into native habitats and infesting them with larvae, we silenced larval MsCYP6B46 expression and observed the behavior of larval predators. The attack behavior of a native wolf spider provided the key to understanding MsCYP6B46's function: the spiders clearly preferred CYP6B46-silenced larvae, just as they had preferred larvae fed nicotine-deficient plants. Additional experiments demonstrated that MsCYP6B46 allows a small amount (0.2%) of the ingested nicotine to move from midgut to hemolymph, providing larvae a means of exhaling nicotine into the headspace through their spiracles as a volatile anti-spider signal. Other abundant native predators which lack the spider's prey-assessment behavior (big eyed bugs and ant lion larvae) were insensitive to the larvae's ingested nicotine, suggesting that nicotine-externalization was specifically effective against spiders. This work demonstrates how plant chemical defenses, too toxic to be readily sequestered and largely excreted through the solid waste stream, can be repurposed for defensive functions through the respiratory waste stream as a form of defensive halitosis.

Manduca sexta | nicotine | Nicotiana attenuata | CYP6B46

Plants produce a pharmacopeia of potent chemical defenses that prevent the attack of unadapted herbivores and thwart the growth of adapted ones. Adapted herbivores tolerate these poisons and repellants by various means (1); some specialist herbivores even co-opt these diet-acquired poisons for their own defensive purposes. When herbivores evolve insensitive target sites, as with the single amino acid substitution in the α-subunit of the Na+/K+-ATPase gene which confers cardenolide tolerance in leaf beetles (2, 3), or avoid metabolizing protoxins into toxins, a plant's chemical defenses can be sequestered by an herbivore and used defensively against its natural enemies (4). The aposematically colored Utetheisa ornatrix moth advertises its sequestered protoxin pyrrolizidine alkaloids and males of the species make further use of this diet-derived defense by metabolizing a part of their defensive stockpile into a pheromone, which during courtship, females use to evaluate the size of a potential mate's defensive endowment that could contribute to the defense of their eggs, after copulatory transfer (5, 6).

Other plant defenses, by virtue of their ubiquitous toxicity are difficult to sequester and co-opt for defense, even by the specialist herbivores. The pyridine alkaloid nicotine is a defense metabolite of several *Nicotiana* spp. It is extremely effective against herbivores due to its ability to poison the essential neuromuscular junction common to all animals that use muscles to move: the acetylcholine receptor (7, 8). However, *Manduca sexta,* the specialist lepidopteran herbivore that feeds on nicotine-producing *Nicotiana* plants tolerates doses of nicotine that are lethal for unadapted herbivores (9). The exact mechanisms responsible for this tolerance remain unclear but both efficient excretion and metabolism appear to be involved. Some researchers have focused on the polar metabolites of nicotine, such as cotinine and the N-oxides of both nicotine and cotinine which have been found in the urine and blood of human smokers (9-11); cytochrome P450s (CYPs) are thought to mediate nicotine's oxidation to these metabolites (9, 10, 12-14). Other researchers propose that nicotine is rapidly excreted without modification (15, 16).

Although *M. sexta* efficiently excretes nicotine, most studies have not been able to recover all of the ingested nicotine in the frass and nicotine can be found in the hemolymph of larvae feeding on nicotine-containing diets. While some studies have reported the polar metabolites in the frass, these experiments either injected nicotine into the hemolymph at high concentrations (9) or forced unadapted larvae to feed on diets with unrealistically high nicotine contents (0.75- 1.0% of fresh diet) (9, 10). The coding sequence of the larvae's acetylcholine receptor proteins does not differ from those of nicotine-sensitive species (9) and an efficient blood-brain barrier is clearly involved, as its nervous system is exposed to nicotine levels that are lethal to unadapted insects (8, 17). Injecting larvae with nicotine or switching unadapted larvae to high nicotine diets results in convulsions and narcosis, until their tolerance machinery can again cope with the detoxification/clearance process (13, 18, 19). These tolerance mechanisms can be demanding; the metabolic load of nicotine tolerance is associated with reduced growth and ingestion (20). *Nicotiana* spp hostplants respond to damage with large increases in nicotine accumulation (21) and the tolerance machinery in

Significance

Hornworm larvae are highly nicotine-tolerant. When they feed on their tobacco host-plants in native habitats, more disappear at night when they feed on host-plants transformed to be nicotine-free, because wolf spiders selectively prey on nicotine-free larvae. When larvae feed on nicotine-replete plants, their, midgut CYP6B46 is strongly upregulated; when this gene is silenced by plant-mediated RNAi, even nicotine-ingesting larvae become spider prey. CYP6B46 expression allows ingested nicotine to pass from midgut to hemolymph, to be exhaled from the spiracles during spider attack. Spiders are deterred by this nicotine-rich halitosis, demonstrating how CYP6B46 functions to repurpose normally excreted nicotine for defense.

Reserved for Publication Footnotes

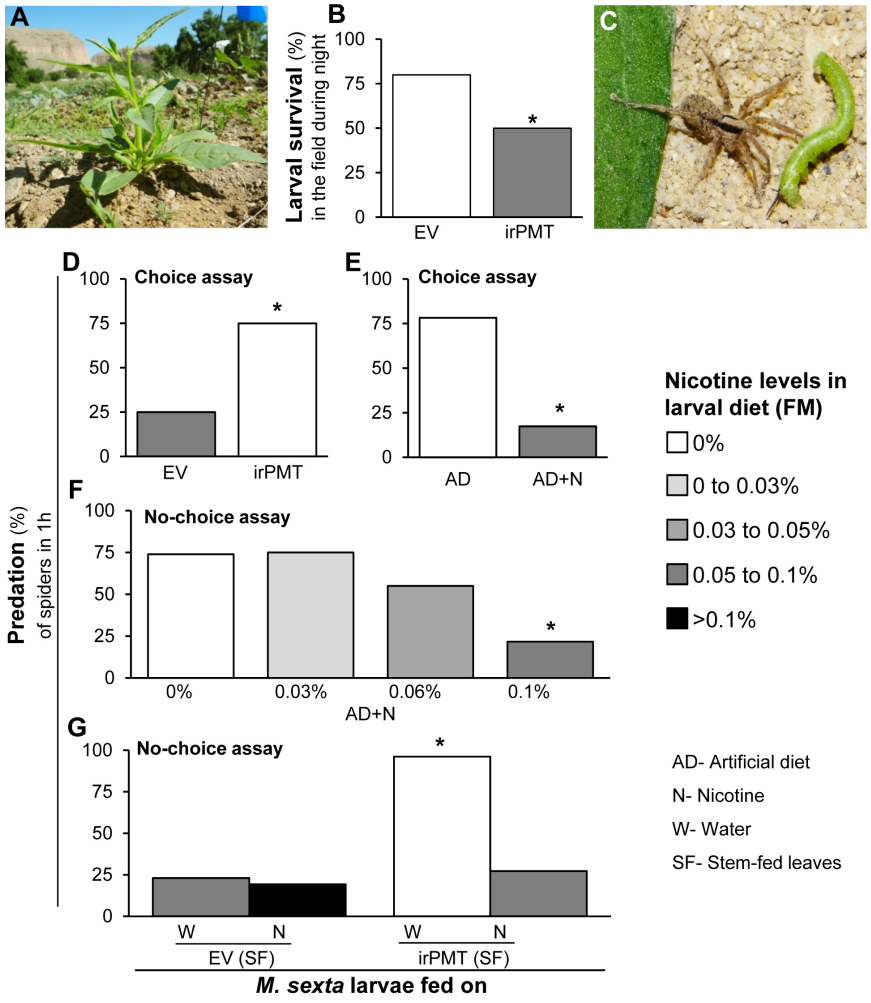

Fig. 1. Spiders are deterred by nicotine-fed larvae (*A*) *Nicotiana attenuata* growing in the Great Basin Desert, Utah. **(*B*)** Nocturnal survival (%) of larvae feeding on nicotine-containing empty vector (EV) and nicotine deficient (irPMT) plants in the field (n=50 larvae per line). **(*C*)** Spider attacking *M. sexta* larva. Spider predation (%) in the choice assay (1h) on 2nd instar *M. sexta* larvae feeding on (**D**) EV(n= 16) and irPMT (n= 16) plants and **(*E*)** AD (n= 23) and AD containing 0.1% of nicotine (AD+N) (n= 23). Spider predation (%) in no-choice assays (1h) with *M. sexta* larvae feeding on: **(*F*)** AD containing 0 (n= 23), 0.03 (n= 20), 0.06 (n= 20) and 0.1% (n= 23) nicotine (AD+N) **(*G*)** water (W) or 1mM nicotine (N) stem-fed (24h) EV and irPMT leaves (n= 26 in all the treatments). Asterisks indicate significant differences ($P \leq 0.05$) by Fisher's exact test on frequencies (and not on the displayed percentages in the figures). **Shading of the bars reflects relative nicotine concentration of the larval diet throughout all figures. Hence, the bar shading provides the important information for the interpretation of transcripts, larval nicotine excretion and the hemolymph-nicotine data presented in the subsequent figures.**

the larvae is regulated by the amount of nicotine ingested (20). Hence within the physiological limits of *M. sexta*'s intake-adjusted excretory-based tolerance lie opportunities for the defensive use of nicotine, particularly against endoparasitoids, which develop within larval tissues.

Indeed more *Cotesia congregata* endoparasitoids emerged as adults from parasitized *M. sexta* larvae fed on low nicotine varieties of cultivated tobacco than from larvae fed nicotine-rich varieties (22). The generalist predatory ant *Iridomyrmex humilis* also preferred *M. sexta* larvae reared on artificial diets (AD) without nicotine over those reared on high nicotine diets and were deterred by topical nicotine treatments (23). Thus *M. sexta* larvae might be able to use this diet-derived toxin for its own protection by virtue of its tolerance machinery, despite a lack of clear sequestration and storage of this toxin.

Here we examine whether *M. sexta* larvae co-opt diet-ingested nicotine for their own defense and whether their detoxification system is involved. In a previous unbiased microarray study, we found that a particular midgut-expressed CYP6B46 was strongly down-regulated in larvae fed hostplants genetically modified to silence nicotine production (24). To examine the function of this CYP, we used a reverse genetics approach, plant-mediated RNA interference (PMRi) (25, 26), to silence this gene in larvae feeding on nicotine-containing *N. attenuata* plants harboring the CYP6B46-silencing construct. These PMRi plants were planted into the native habitat of both hostplant and larvae, the Great Basin Desert, Utah, USA which teems with larval predators, such as bugs, mantids, ants, ant lions, spiders and lizards. One of these predators, a wolf spider, selectively predated CYP6B46-silenced larvae, just as they did larvae feeding on nicotine-free hostplants. The particular predatory behavior of the spiders revealed the function of *Ms*CYP6B46 in externalizing ingested nicotine for defensive use.

Results

Wolf spiders avoid nicotine-fed larvae in nature

To investigate the effect of hostplant nicotine on the survival of *M. sexta*, we transplanted stably transformed *N. attenuata* plants silenced in nicotine production and accumulation [inverted repeat (ir)PMT] (27) into a field plot in Utah (Fig. 1*A*). Survival rates of *M. sexta* larvae feeding on nicotine-deficient and -producing plants in the predator-rich field were monitored and we found that fewer irPMT-fed larvae survived than did larvae feeding on control plants producing WT levels of nicotine [empty vector (EV)-fed], especially during the nights (Fig. 1*B*; Table S1); during the day, no significant differences in survivorship were found [EV= 76%; irPMT= 72% (n=50 larvae per line)]. We hypothesized that this difference in survivorship resulted from the selective predation of a night-active nicotine-sensitive predator. We found wolf spiders [*Camptocosa parallela* (Lycosidae)] during night-time surveys of the *N. attenuata* field plantation (Fig. 1*C*) (density 1.55± 0. 05 individuals/m^2). We tested these spiders in choice and no-choice assays (Fig. S1*A*- *C*) with *M. sexta* larvae fed on foliage or artificial diet (AD) of different

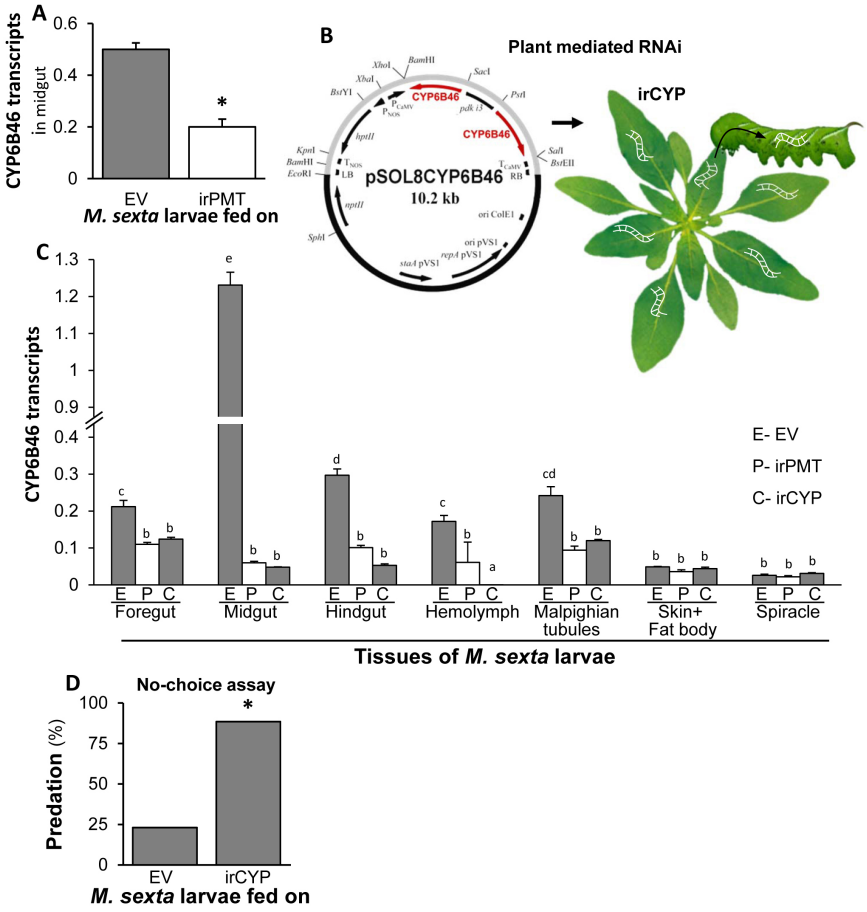

Fig. 2. Silencing larval CYP6B46 dramatically affects spider predation(*A*) CYP6B46 transcript levels (relative to ubiquitin) in midguts of 1st instar larvae feeding on WT and irPMT *N. attenuata* plants ($F_{1,8}$= 9.984, P≤0.05, n=5). **(*B*)** Schematic representation of plant mediated RNAi: pSOL8 binary vector constructed to express 300bp dsRNA of *Ms*CYP6B46 in *N. attenuata* and trophic transfer of CYP6B46 dsRNA from plant to larvae **(*C*)** CYP6B46 transcript levels (relative to ubiquitin) in various tissues (foregut, midgut, hindgut, hemolymph, Malpighian tubules, cuticle with fat body and spiracle) of 4th instar larvae feeding on EV (E), irCYP (C) and irPMT (P) plants ($F_{20,84}$= 487.2, P≤0.0001, n=5). **(*D*)** Spider predation (%) in no-choice assays on larvae fed EV and irCYP leaves (n= 26). Asterisks and small letters above the bars in *A* and *C* indicate significant differences determined by one-way ANOVAs; asterisks in *D* and *E* indicate significant differences (P≤0.05) by Fisher's exact test. See Fig. 1 legend for the codes for the bar-shading.

nicotine contents (Fig. 1*D- F*). *C. parallela* strongly preferred larvae fed irPMT plants over those fed EV plants and similarly, AD-reared larvae over those reared on nicotine-containing AD (Fig. 1*D- F*). The nicotine-sensitivity of this spider was confirmed in no-choice assays, in which the predation rate was found to decrease with increasing nicotine concentrations in the larval diet (depicted by the shading of the bars in all figures) (Fig. 1*F*). Larvae fed nicotine-supplemented [stem-fed (Fig. S1*D*)] irPMT leaves (irPMT+N) were predated at rates similar to those of larvae on plants with WT-levels of nicotine (Fig. 1*G*). From these results, we conclude that the spiders were deterred by *M. sexta* larvae's ingested nicotine or a metabolic product thereof.

Silencing *M. sexta's* nicotine-induced CYP6B46 by PMRi

That midgut CYP6B46 transcript accumulation was elicited specifically in response to nicotine ingestion was confirmed using larvae fed control (EV plants with WT nicotine levels) and nicotine-deficient irPMT plants (Fig. 2*A*) and further confirmed in additional experiments with larvae fed irPMT plants stem-fed water (irPMT+W) or nicotine solutions (irPMT+N) (Fig. S2*A*) and AD lacking nicotine or AD containing 0.1% nicotine (AD+N) (Fig. S2*B*). To understand *Ms*CYP6B46's function in larval nicotine metabolism, we created a transgenic line of *N. attenuata* to silence the expression of CYP6B46 in *M. sexta* larvae feeding on these plants, using plant mediated RNA interference (PMRi) (25, 26).

We transformed *N. attenuata* plants with the recombinant vector containing two 300bp fragments of *Ms*CYP6B46 in an ir orientation to create stable transgenic irCYP6B46 (irCYP) plants (homozygous for a single genomic insertion) that synthesized double stranded RNA (dsRNA) of *Ms*CYP6B46 under control of a strong CaMV promotor (Fig. 2*B*). irCYP plants were indistinguishable from isogenic WT plants in growth and morphology and most importantly, had nicotine contents equivalent to those of WT plants when grown in both the field and glasshouse (Fig. S2*C*). When *M. sexta* larvae ingested leaves from these irCYP plants and consequently CYP6B46 dsRNA (Fig. 2*B*), dramatic (95%) sequence-specific silencing of larval CYP6B46 was observed in their midguts, the tissue with the highest transcript accumulation levels (Fig. 2*C*). Transcript abundance was also significantly reduced in the foregut, hindgut, hemolymph, and Malpighian tubules, which had much lower basal levels of expression than did the midguts (Fig. 2*C*). While strong silencing was observed in internal larval tissues, silencing of the very low basal levels of expression in the skin, fat body and spiracles was not evident, suggesting that the PMRi procedure may be most effective in tissues most exposed to ingested leaf material. These results clearly demonstrated that larvae feeding on irCYP plants were strongly silenced in their midgut CYP6B46 expression, and for brevity, we refer to these as "CYP-silenced" larvae.

In situ CYP6B46 silencing increases larval susceptibility to predatory spiders

We planted irCYP plants into a field plot in the plant's native habitat, infested them with larvae and compared larval survivorship with those infesting WT control plants. CYP-silenced larvae survived similarly poorly on these plants as normal larvae had on the nicotine-deficient irPMT plants during night time [EV= 80%; irCYP= 50% (n=50 larvae per line)]. We hypothesized that this difference was due to the selective predation of the nicotine-sensitive wolf spiders. In no-choice assays with larvae fed individually on irCYP and EV plants, spiders consumed

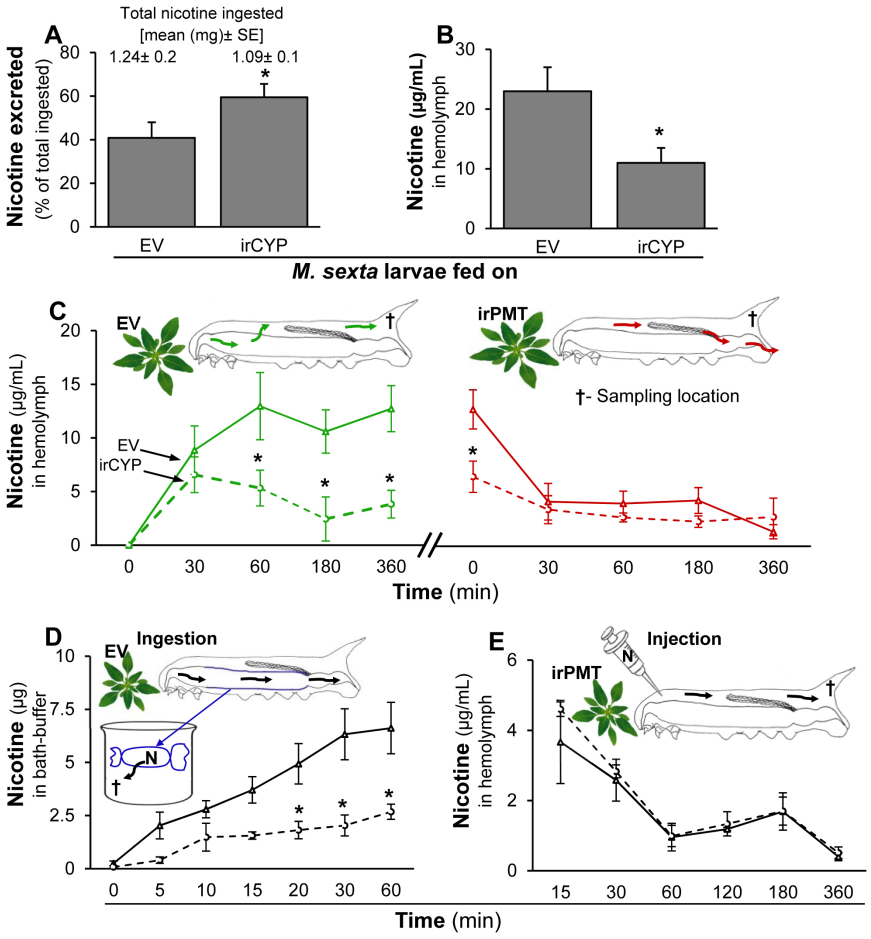

Fig. 3. Effect of CYP6B46 silencing on larval nicotine excretion and nicotine flux in larval body(*A*) Nicotine excreted (% of total ingested) by 4th instar EV or irCYP feeding larvae (experimental details in Fig S3*A*) [(mean± SE) $F_{1,12}$= 8.77, P≤0.05, n=8]. **(*B*)** Nicotine in hemolymph of 4th instar EV or irCYP feeding larvae ($F_{1,4}$= 106.6, P≤0.01, n=5). **(*C*)** Kinetics of nicotine absorption by (green arrows in larval body and green lines in graph) and discharge from (red arrows and red lines) the hemolymph of control and CYP-silenced 4th instar larvae (experimental details in Fig S3*B*). **(*D*)** Kinetics of nicotine discharge from excised midguts (containing ingested host-plant diet) of control and CYP-silenced 4th instar larvae; nicotine entering the bathing solution (sodium phosphate buffer+ 0.3M sorbitol, pH 7.0) was measured at regular intervals, up to 60min. **(*E*)** Kinetics of nicotine discharge from hemolymph of control and CYP-silenced 4th instar larvae, after injecting 0.001% nicotine (of FM) into the hemolymph to determine if CYP-silenced larvae discharge nicotine from their hemolymph at rates different from that of controls (experimental details in Fig. S3*B*). Asterisks indicate significant differences determined by one way ANOVA (P≤0.05). See Fig. 1 legend for the bar-shading codes.

significantly more CYP-silenced larvae than control larvae (Fig. 2*D;* Video S1 *A* and *B*). These bioassay results suggested a role of nicotine ingestion and CYP6B46 expression in *M. sexta*'s spider-deterrence.

CYP6B46's role in *M. sexta*'s processing of nicotine

The spider's predation behavior clearly established an association between CYP6B46 expression and *M. sexta*'s spider-deterrence abilities. To evaluate this association, we quantified the previously reported (9, 10) oxidation products of nicotine [cotinine, cotinine N-oxide (CNO) and nicotine 1-N-oxide (NNO)] in the frass of control and CYP-silenced larvae. We developed a sensitive and accurate U(H)PLC-micrOToF mass spectrometer based procedure using internal standards to quantify nicotine, cotinine, and their N-oxides with a limit of detection of 0.25ng (nicotine) and 0.5ng (cotinine, CNO and NNO) and a high efficiency of extraction (>90%) of these compounds from frass (Fig. S3*A-D*). As had been previously reported by Self et al. (16) almost 50 years ago, we found no evidence for metabolites other than nicotine in the frass.

Since nicotine oxidation products were not found, we hypothesized that the food intake of the CYP-silenced larvae was lower than the controls thereby lowering the overall food- and consequently nicotine-content of their body and making them susceptible to the spiders. To test this, we adopted the mass-balance approach of the Waldbauer assays (28, 29) to quantitatively evaluate the flux of nicotine through larval bodies. We compared the mass of food and nicotine ingested and excreted by the CYP-silenced and EV-fed larvae, which were not different (Fig. S3*E*; Table S2). However, we observed that during this 24h assay, although the EV- and irCYP-fed larvae ingested the same amount of nicotine in the foliage they consumed, CYP-silenced larvae excreted significantly more (~20%) in their frass than did EV-fed control larvae (Fig. 3*A*; Table S2).

CYP6B46 silencing alters nicotine efflux from midgut to hemolymph

The results of the nicotine-Waldbauer assays revealed that CYP-silenced larvae retained less nicotine in their body and excreted more in their frass. We examined larval hemolymph and found that the hemolymph of CYP-silenced larvae contained 47% less nicotine than did the controls (Fig. 3*B*). We developed a procedure to multiply sample the hemolymph of individual larvae as they fed first on nicotine-free irPMT plants, were switched to nicotine-containing EV plants, and were returned back onto irPMT plants to compare the nicotine dynamics in the hemolymph of control and CYP-silenced larvae as they ingested a realistic doses of dietary nicotine (Fig. S4*A* and *B*). Previous work had shown that larvae clear a majority of ingested or injected nicotine within 6h (16) and this time interval was used between the dietary switches.

Hemolymph nicotine levels rapidly increased after larvae were switched to nicotine diets to attain steady state levels within 30-60min. CYP-silenced larvae attained steady state levels that were ~70% lower than those of EV-fed larvae [after 360min, EV: 12.7± 2.1 (mean± SE) µg/mL and CYP-silenced: 3.9± 1.2 (mean± SE) µg/mL] (Fig. 3*C*). When switched back to nicotine-free plants, hemolymph values of both larval types returned to similar levels within 30min (Fig. 3*C*). These results demonstrate that CYP6B46 expression dramatically influences the flux of

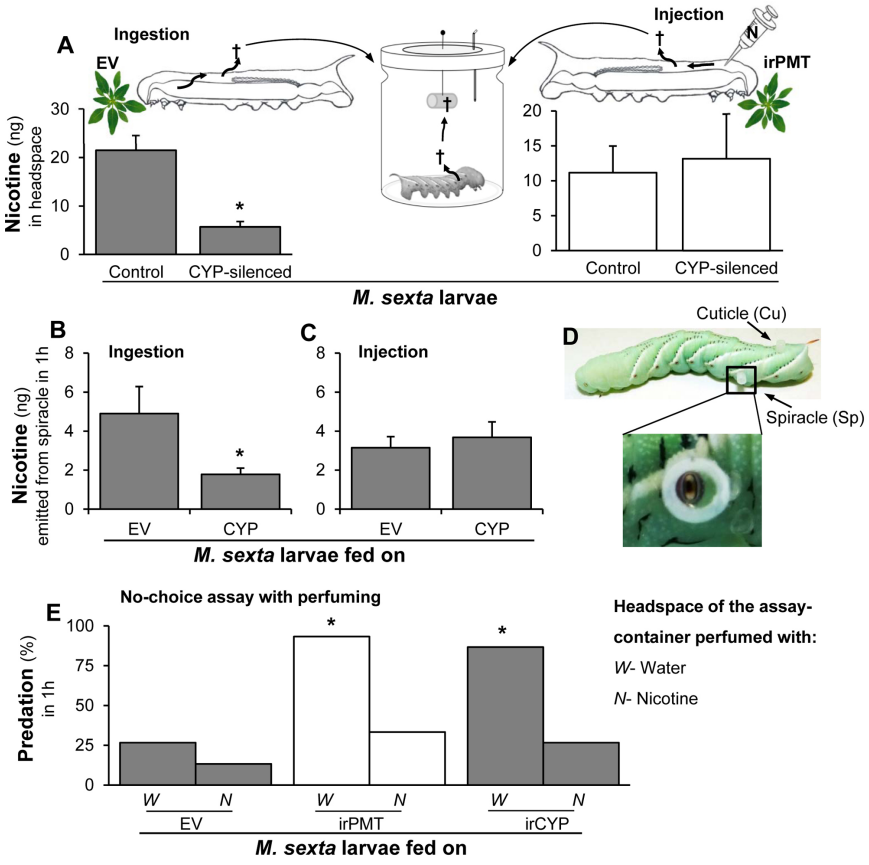

Fig. 4. CYP6B46 silencing reduces larval nicotine emission and increases spider predation, which can be complemented by volatile nicotine perfuming(*A*) Emission of ingested nicotine (left bar chart) [(mean± SE) $F_{2,6}$= 36.14, P≤0.0005, n=3] by the 4th instar larvae feeding on EV and irCYP plants and emission of injected nicotine (right bar chart) [(mean± SE) n=3] by the nicotine-free control and CYP-silenced 4th instar larvae. Nicotine adsorbed on the PDMS tube attached to a spiracle of the 4th instar control or CYP-silenced larvae (each weighing 7.0± 0.25g): **(*B*)** after feeding on nicotine containing leaves for 1h [(mean± SE) $F_{1,9}$= 5.82, P≤0.05, n=5] or **(*C*)** after injecting 0.001% nicotine (of FM) [(mean± SE) n=5].**(*D*)** *M. sexta* larva with attached PDMS tubes for the volatile nicotine trapping from the spiracle (Sp) and cuticle (Cu). **(*E*)** Spider predation (%) in a no-choice assay on *M. sexta* larvae fed on EV, irPMT and irCYP plants with water or nicotine perfuming (n= 15/treatment); experimental details are given in Fig. S4*C*. Asterisks above the bars in *A- C* indicate significant differences determined by one way ANOVA; asterisks above the bars in E indicate significant differences (P≤0.05) by Fisher's exact test. See Fig. 1 legend for the bar-shading codes.

nicotine into the hemolymph from the ingested food, but not its clearance from the hemolymph once the ingestion ceases.

To further examine both inferences, we conducted two additional experiments. In the first, size-matched control and CYP-silenced larvae that were fed EV plants for 6h before their midguts were dissected, sutured and sealed at both the ends without loss of gut contents and incubated in a bath containing a neutral pH buffer (matching hemolymph) for 60min (Fig. 3*D*). Midguts of CYP-silenced larvae released ∼60% less nicotine into the bath buffer, compared to midguts of control larvae (Fig. 3*D*); moreover, none of the polar nicotine metabolites were detected in the bath buffer, suggesting an important role for CYP6B46 in the transfer of nicotine into the hemolymph. To test the second inference about CYP6B46's lack of role in the clearance of nicotine from the hemolymph, we bypassed the midgut associated function of CYP6B46 by injecting a physiologically realistic quantity of nicotine directly into the hemolymph of control and CYP-silenced larvae feeding on nicotine-free plants (Fig. 3*E*). Again, we found no significant difference in the clearance of nicotine from the hemolymph of both groups of larvae over 6h (Fig. 3*E*) and again no evidence of polar nicotine metabolites, consistent with the results of the previous feeding experiment.

Spider predatory behavior revealed that larvae externalize ingested nicotine

The nicotine levels in the hemolymph of CYP-silenced larvae were clearly lower than those of control larvae feeding on the same nicotine-containing diets; could the lower hemolymph nicotine levels account for the large increase in nicotine excretion in the frass? In 24h, when both normal and CYP-silenced larvae consumed ∼1.1 mg of nicotine in their diets, CYP silenced larvae excreted ∼0.13mg more nicotine than did control caterpillars in their frass (Table S2). The amount of nicotine in hemolymph of a control larva is only 23± 4.0µg/mL (mean± SE); considering that the larvae used for the Waldbauer assays weighed 1± 0.25g (mean± SE) and contained 300± 60µL (mean± SE) hemolymph, the hemolymph could only account for ∼7.2 µg nicotine. Thus, retention of the large amount of unaccounted for nicotine (∼740µg in control and ∼370µg in the CYP-silenced larvae) by the hemolymph or its excretion through the hemolymph during a 24h feeding period, seemed unlikely. Hence we returned to our observations of spider predatory behavior for a clue about the missing nicotine.

Spiders usually assess their prey after capture by tapping it with chemosensory endowed legs and palps (30) (Video S1*A- C*). Wolf spiders were clearly rejecting nicotine-fed larvae before penetrating their prey with their mandibles; this suggested that larvae externalize some fraction of their ingested nicotine. We washed larvae feeding on different nicotine-containing diets and found no evidence for surface externalization of the ingested nicotine that couldn't be attributed to direct surface contamination (Fig. S5*A*). This motivated us to explore if larvae emit some fraction of their ingested nicotine into the headspace. We analyzed the headspace of these larvae and found significant quantities of nicotine. We compared the headspaces of control and CYP-silenced larvae that had ingested the same amount of nicotine in their food and found that the headspace of control larvae contained 4-fold more nicotine [22± 3.0ng (mean± SE)] than the headspace of CYP-silenced larvae [5± 1.0ng (mean± SE)] (Fig. 4*A*, S5*B* and S5*C*).

CYP6B46-silencing inhibits the spiracular release of nicotine and makes larvae vulnerable to spider predation

To evaluate if the headspace reflected the differences in hemolymph nicotine observed between CYP-silenced and control larvae, we injected nicotine into their hemolymph of both control and CYP-silenced larvae after rendering them both nicotine-

free by feeding them on irPMT plants; the headspace nicotine levels of these injected larvae did not differ significantly between control and CYP-silenced larvae and were in the same range as normal nicotine-fed larvae (Fig. 4*A*). Again, none of the nicotine-metabolites could be detected in the headspace of larvae.

To understand how hemolymph nicotine levels could translate into headspace emissions, we developed a procedure to specifically quantify nicotine emissions from larval spiracles, the mouth equivalents of a caterpillar and the lung equivalent, the tubular tracheal system that ramifies throughout the larvae body supplying oxygen via microscopic tracheoles. We glued small segments of polydimethylsiloxane (PDMS) adsorptive tubes either directly over spiracles or onto the adjacent skin (Fig. 4*B*) and quantified nicotine emissions at different times from AD fed larvae into which we had just injected nicotine into their hemolymph. Nicotine emissions from the spiracles increased dramatically, tracking the expected increase in hemolymph concentrations (Fig. S6*A*).

We next compared spiracular nicotine emissions of control and CYP-silenced larvae and found that the difference in headspace emissions corresponded to their spiracular emissions (Fig. 4*C*). To test whether this spiracular emission was controlled by CYP6B46 activity in larval midguts, we injected nicotine into CYP-silenced and control larvae to equalize their hemolymph nicotine concentrations and found their spiracular nicotine emissions to be equivalent (Fig. 4*D*).

Lastly, to evaluate whether the difference in spiracular nicotine emission could account for the spider feeding preferences, we conducted no-choice assays in which the headspaces of irPMT, irCYP, and EV-fed larvae were perfumed with water or amounts of nicotine that were only 5 times that found in larval headspace that accumulates in a 50mL chamber enclosing one larva for 1h (Fig. S6*B* and *C*). This concentration is likely a conservative estimate of the amount of nicotine emitted by the larvae during a spider-prey encounter. Indeed, when the headspace of spider-preferred irPMT- or CYP-fed larvae (which were not emitting nicotine beforehand) was nicotine perfumed, spider predation decreased by 64% ($P \leq 0.05$) (Fig. 4*E*). Clearly, dietary nicotine is only used defensively by larvae with a fully active CYP6B46. A similarly negative effect on the spider's predatory behavior was also observed in assays performed with AD-fed larvae with and without headspace perfuming (Fig. S6*D*).

Other abundant predators are not deterred by *M. sexta*'s dietary nicotine

Apart from spiders, many other predators frequently explore *N. attenuata* plants in its native habitat. It is plausible that *M. sexta*'s nicotine exhaling mechanism could be effective against a broad spectrum of predators. The most frequently observed diurnal predator in the field plot is the big eyed bug *Geocoris pallens* (Lygaeidae) (31, 32) (3.7± 0.1 individuals/m^2 in 2013). To evaluate the effect of dietary nicotine on *G. pallens* predation, we conducted no-choice assays in which EV-, irPMT- or irCYP-fed second-instar larvae were offered to *G. pallens*; these diurnal predators did not differentiate between the larvae fed on different plants (Table S3).

Another locally abundant generalist predators are ant lion larvae [*Myrmeleon carolinus* (Myrmeleontidae)]. They construct funnel-shaped pits in sandy soil adjacent to *N. attenuata* plants in the field plantation and are known for their ability to avoid toxin-containing tissues of their prey (33). Since nicotine is present throughout the body of *M. sexta* larvae, we hypothesized that ant lions would reject larvae fed nicotine-containing diets. EV-, irPMT- and irCYP-fed second instar larvae were dropped into separate ant-lion pits and their response (feeding or rejection) was recorded. These predators also did not differentiate between larvae fed on the different diets (Table S3).

Discussion

Most biologists will agree that understanding the function of genes is a central research objective for the field and that ultimately, genes function at the level of the organism, where their influence on an organism's Darwinian fitness determines whether a gene will be retained in genomes or pseudogenized over evolutionary time. Querying the transcriptomes of organisms as they respond to environmental challenges can provide valuable hints for the genetic basis of adaptations, and the various reverse genetics tools that allow researchers to silence candidate gene expression, has provided one of the most successful means of understanding gene function at the organismic level (25). In many research programs, the analysis of gene function starts with an understanding of a gene's biochemical function, and here heterologous expression studies play a central role as substrates and products are most revealing. However, negative results from such assays are difficult to interpret and biochemical inactivity of the heterologously expressed protein will terminate a research program, just as a lack of biochemical or physiological phenotype in a 'loss of function' system does, frequently causing researchers to adopt the fallback hypothesis of "gene redundancy", when the gene is a member of a larger gene family. Moreover, such negative results frequently struggle to find a place in the mainstream literature, as was the case with RNAi functional genomic studies in lepidopteran insects (34).

Cytochrome P450 genes are ubiquitous and occur in large families (35). They play important ecological and evolutionary roles in the interactions of plants and their attackers, as they are frequently involved in the detoxifications of diverse xenobiotics by mediating the reactions like N- or S-oxidation, hydroxylation, epoxidation and O-, N- or S- dealkylation (36, 37). The particular CYP that we found to be strongly regulated in response to dietary nicotine intake in *M. sexta* larvae, *Ms*CYP6B46, is a member of the CYP6B enzyme family, which is well known for its roles in the detoxification of plant defenses, and appears to have diversified in lepidopteran taxa with more diverse diets than those with more specialized diets (38-40). Our functional analysis of *Ms*CYP6B46 stumbled after not finding the expected polar metabolites of nicotine in larval tissues and frass or from heterologous expression assays, but was revived when the behavior of a native predator revealed an unexpected organismic level function of *Ms*CYP6B46.

The challenges of phenotyping organisms whose natural history we do not understand well frequently limit our ability to identify phenotypes in gene-silenced or KO organisms. However, all organisms in their natural environments, and particularly plants, as they lie at the base of all terrestrial food chains, are carefully scrutinized by literally thousands of other organisms with very different, frequently highly hostplant-tuned sensory modalities. Hence the phenotyping services that competitors, pathogens, herbivores, pollinators, predators and the plethora of different types of mutualists that interact with an organism lacking the expression of a particular gene, allow for an unbiased 'ask the ecosystem' approach to understanding the function of this gene at an organismic level. Planting the nicotine-deficient irPMT *N. attenuata* plants into the field plots identified a nicotine-sensitive predator, the wolf spider, and the particular prey assessment behavior of this predator when attacking *M. sexta* larvae clearly showed that silencing *Ms*CYP6B46 expression interfered with the larvae's ability to externalize a defense metabolite, which provided the entry point into discovering *Ms*CYP6B46's role in how *M. sexta* larvae process the large quantities of nicotine that it ingests each day.

M. sexta larvae silenced in CYP6B46 expression were attacked and consumed by *C. parallela* spiders even though they consumed equivalent amounts of nicotine compared to control larvae (Fig. 2). Thus the processing of ingested nicotine within the larvae and not the higher nicotine content in the frass, which

we later discovered in CYP-silenced larvae, were responsible for the ability of control larvae to ward off spider attack. While all three predators examined in this study, *G. pallens,* ant lion larvae and wolf spiders, kill their prey by injecting a cocktail of digestive enzymes and toxins before sucking out their prey, wolf spiders include a prey-assessment step, during which the spider hovers over the prey, tapping the prey with chemosensory endowed legs and palps, before piercing the prey (*35*) (Video S1*A- C*). Recently, we discovered that *M. sexta* larvae emit the branched chain aliphatic acids by hydrolyzing the *O*-acyl sugars that they acquire from the glandular trichomes of *N. attenuata* (41), which motivated us to explore how larvae might externalize some fraction of their ingested nicotine and led to the discovery of *Ms*CYP6B46's role allowing ingested nicotine to pass from midgut to hemolymph to be exhaled from the spiracles during spider attack. While budgeting the nicotine flux through the larvae, we found that CYP-silenced larvae had lower hemolymph- and higher frass- nicotine levels, so we examined the dynamics of nicotine in the hemolymph after switching larvae from or to the nicotine-containing foliage. CYP6B46 mediated the transition of nicotine from the guts into the hemolymph, but not the clearance of nicotine from the hemolymph (Fig. 3). Although the function of CYPs have been mainly proposed to mediate the oxidation of nicotine (10, 13), none of the nicotine oxides were detected in hemolymph, frass, excised guts and larval headspace of WT fed and CYP silenced larvae [which is consistent with the findings of Self et al (16)]. The midgut based CYP6B46 may convert nicotine to short-lived metabolite that is readily pumped, which rapidly reverts back to nicotine after entering hemolymph, as originally proposed by Morris (42).

Malpighian tubules and blood brain barrier of *M. sexta* larvae contain pumps that clear the nervous system of alkaloids such as nicotine, morphine and atropine (43, 44); these pumps also purge the Malpighian tubule lumen of the alkaline synthetic dyes (26). A similar pump may also distribute nicotine from midgut to hemolymph. Considering the biochemically validated functions of insect CYPs (10, 24, 33), it is unlikely that CYP6B46 itself acts as a nicotine-pump, but it could be part of a multicomponent system that includes transporters that could use the large midgut-hemolymph pH gradient as a driving force. However, a much more powerful pump must be involved in retaining such a large part of the ingested nicotine in the digestive tract and clearing nicotine from the hemolymph and nervous system (8, 42).

This work has demonstrated that *M. sexta*'s nicotine tolerance is clearly based on efficient excretion and that the function of *Ms*CYP6B46 counters this remarkably efficient excretion system and allows nicotine to pass from the larvae's digestive system into the hemolymph by an unknown mechanism, which in turn provides a means of externalizing nicotine into the larvae's headspace. In this scenario, *M. sexta*'s defensive halitosis, evolved to repurpose waste from an efficient means of excreting unsequesterable toxins from their food. Whether this role of *Ms*CYP6B46 in re-purposing nicotine for a defensive function was a secondary innovation evolving after the basic excretory machinery was in place, could be explored in other nicotine-tolerant taxa within the sphingid clade by studying the role of this CYP in different species. Whether *Ms*CYP6B46 is also responsible for the differential survival of endoparasitoids (45) and for the selective predation by ants (23) is also an intriguing possibility deserving further study. It will also be interesting to see if the other nicotine-tolerant relatives of *M. sexta* that normally feed hostplants that lack nicotine (9) have evolved similarly functioning orthologs of CYP6B46.

Materials and methods

Plant material

Nicotiana attenuata 30x inbred seeds, which were originally collected in 1988 from a native population at Utah, USA were used for the generation of *Agrobacterium tumefaciens*-mediated stable transgenic lines by the procedure described in Krügel *et al* (46). Seeds were germinated on sterile Gamborg B5 medium (Duchefa, Harleem The Netherlands) after 1h of treatment with diluted smoke (House of Herbs, Passaic, NJ) and 1μM GA_3 (Roth, Karlsruhe Germany) (46). Ten days after germination, seedlings were transferred into Teku pots containing a peat-based substrate, and after an additional 10 to 12d, the plantlets were transplanted into individual 1L pots with the same substrate. In the glasshouse, plants were grown at 24°C to 26°C, relative humidity approximately 60%, and supplemented with light from 400- and 600-W sodium lamps (Philips, Herrsching Germany) for 16h (47).

irPMT (*Na*PMT NCBI accession no. AF280402) *N. attenuata* plants (A-03-108-3) fully characterized in (27) was used as a nicotine-free hostplant to feed *M. sexta* larvae. Previously characterized *N. attenuata* transgenic line irCYP (A-09-30-2) was used to silence *M. sexta*'s CYP6B46 (*Ms*CYP6B46 NCBI accession no. GU731529) by PMRi. Generation of irCYP line and silencing of *M. sexta* larval genes was reported previously (Fig. 2*B*) (26). EV transformed plant line (A-04-266-3) was used as transgenic control plant (48).

Field experiments were conducted at Lytle Ranch Preserve in Santa Clara, Utah, 84765 (37°08'45"N, 114°01'11"W) 2004 to June 2013. Seeds of *N. attenuata* irPMT and irCYP lines were imported and released in accordance with several APHIS notifications (Table S4). Planting of transgenic lines in the field plot was performed as described in Kessler *et al* (2012).

***M. sexta* larvae**

Eggs of the in-house reared *M. sexta* were stored in a growth chamber (Snijders Scientific, Tilburg, Netherlands) at 26°C/ 16h light, 24°C/ 8h dark, until the larvae hatched; these larvae were used for all the glasshouse related experiments. For field experiments, *M. sexta* eggs were provided by North Carolina State University (Raleigh, North Carolina, United States) in 2004/2005 and by Dr. Carol Miles (Department of Biological Sciences, Binghamton University, NY, USA) in 2012.

In various experiments, artificial diet (AD) (49) was fed to the larvae. This enabled us to control dietary nicotine concentrations, whenever required; it also enabled us to rear larvae free from any influence of the hostplant.

Survivorship assays with *M. sexta* larvae feeding on EV, irPMT or irCYP plants

In 2013, *M. sexta* larvae were fed on EV, irCYP or irPMT plants until the second instar. Fifty larvae from each *N. attenuata* line were placed on the plants of the same respective line (3 larvae/ plant) that were planted across a predator-rich field in a random spatial array. To quantify survival during the day, larvae were placed on plants in the field at 6am and the number of larvae surviving on each plant was counted at 8pm; to quantify nocturnal survival, larvae were placed on plants in the field at 8pm and surviving larvae were counted at 6am. Survivorship assays were also conducted in 2004 and 2012; their details are given in the supporting information.

Predator abundance in the field

In 2013, predators were counted in $1m^2$ quadrats randomly placed in the field plot; *C. parallela* individuals were counted from 20 quadrats, whereas *G. pallens* individuals were counted from 15 quadrats.

Spider predation assays

C. parallela spiders were collected from in and around the *N. attenuata* field plantation where they were particularly abundant. Spiders were placed individually in chambers and starved for 12h before all assays. Each assay was conducted for up to 1h with late second instar larvae. Spiders were never reused in experiments. Spider's choice or predation was recorded only if the spider consumed the entire larva within the duration of the assay.

(i) *Choice assay*

One test (irPMT/ irCYP/ AD+N fed) and one control (EV/ AD fed) larvae were placed with a single spider inside the polypropylene container (60cc) (Fig. S1*A*). Each spider was allowed only one choice during the 1h assay period. Spiders' choices of larvae were expressed in terms of the percentage of spiders that chose larvae from each test treatment.

(ii) *No-choice assay*

One larva was enclosed with one spider in each assay container (Fig. S2*B;* Video S1*A- C*). Assays with test and respective control larvae were always performed simultaneously. Percentage of larvae preyed on by the spider in 1h was calculated for each treatment group of larvae.

(iii) *No-choice assay with perfuming*

In each assay, two larvae feeding on the same *N. attenuata* line or on the same AD combination were placed in two separate assay containers. Each container contained an Eppendorf tube containing a cotton swab moistened with 500μL of 1mM nicotine (Sigma-Aldrich, Germany) or 500μL water (control) and its opening was covered with perforated parafilm. One spider was placed in each container and was monitored for 1h. For each larval treatment group, the percentage of larvae preyed on by the spider was calculated for each perfuming treatment.

RNA isolation and quantitative real time PCR

One day old larvae were used to examine the changes in CYP6B46 transcript abundance in response to nicotine ingestion. Midguts of these larvae were dissected; midguts of 5 larvae were pooled to produce one sample. Hemolymph, Malpighian tubules, foregut, midgut, and hindgut were collected from the fourth instar larvae, as reported earlier (26); in addition, pieces of larval cuticle (with associated fat bodies) from spiracular and non-spiracular regions were collected. To quantify CYP6B46 silencing

efficiency and conduct tissue-specific CYP6B46 transcript profiling, fourth instar larvae were used.

In the nicotine flux determination experiments, to render the larvae nicotine-free or to feed them the same diet as that of control larvae, fourth instar CYP-silenced larvae were fed (for 6-12h) on irPMT or EV plants, respectively. To evaluate if the CYP silencing persisted in such CYP-silenced non-irCYP feeding larvae, CYP6B46 transcripts were profiled in midguts after both control and CYP-silenced larvae fed on irPMT or EV plants for 24h.

RNA isolation was performed using Trizol reagent (Invitrogen, Germany), according to the manufacturer's protocol. Quantitative real time PCR to measure CYP6B46 transcript levels was performed as reported by Kumar et al (26). Ubiquitin was used as an internal control to normalize the abundance of CYP6B46 transcripts.

Waldbauer assays for nicotine budgeting

Waldbauer assays to budget the ingested and excreted nicotine in control and CYP-silenced *M. sexta* larvae were performed as described earlier (28, 29, 50). Schematic flowchart of Waldbauer assay protocol is shown in Fig. S3*E* (*SI Materials and Methods*).

Kinetics of nicotine flux in larvae

Schematic flowchart of the experimental procedure for the monitoring of the nicotine kinetics in larval hemolymph is shown in Fig. S4*A*. All larvae used in this analysis were in the 4th instar and of similar masses (7.0g± 0.25g). Hemolymph (2µL) of each larva was collected at 0, 30, 60, 180, and 360 min by clipping the tip of the larval horn.

Afflux of ingested nicotine to hemolymph

Controls and CYP-silenced larvae were rendered nicotine-free by feeding them on irPMT plants for 6h. To restart the nicotine flux from midgut to hemolymph, these larvae were fed leaves from EV or irCYP plants (having equivalent nicotine contents), respectively, for 6h and the amount of leaf mass consumed was quantified. Only the larvae that fed continuously during these 6h were used in the analysis. Nicotine concentrations of the collected hemolymph samples (for control and CYP-silenced larvae, for each time-interval, n= 5) were measured by U(H)PLC/ESI-QTOF-MS (micrOTOF QII) (51) (*SI Materials and Methods*).

Efflux of ingested nicotine from hemolymph

Larvae that were used to monitor the nicotine afflux to hemolymph were fed on leaves of their respective plant lines for 24h. They were then transferred to nicotine-free irPMT plants to terminate nicotine ingestion and to begin the gradual clearing of nicotine from midgut and hemolymph. Nicotine concentrations of the collected hemolymph samples (for control and CYP-silenced larvae, for each time-interval, n= 5) were measured by U(H)PLC/ESI-QTOF-MS (micrOTOF QII).

Efflux of injected nicotine from hemolymph

This experiment was conducted for determining the kinetics of nicotine discharged from the hemolymph of control and CYP-silenced larvae, in the absence of the flux of ingested nicotine from midgut into hemolymph. Guts and hemolymph of EV and irCYP feeding larvae were rendered nicotine-free by feeding these larvae on irPMT plants for 6h. Nicotine (70±2.5µg) was injected to the hemolymph of each of these larvae to attain a concentration of 0.001% of FM. Injection volume was constant (50µL) for every larva and the dorsal point between 5th and 6th body segments was used as the injection site. After injection, hemolymph samples were collected (for control and CYP-silenced larvae, for each time-interval, n= 5) their nicotine concentrations were measured by U(H)PLC/ESI-QTOF-MS (micrOTOF QII)

Nicotine efflux from the dissected midgut

In order to understand if the CYP silencing in the midguts of irCYP fed larvae influenced the kinetics of nicotine efflux from the midgut, we conducted the assays using excised midguts of control and CYP-silenced larvae. EV and irCYP fed 4th instar larvae were fed on EV plants for 6h to ensure that their gut-contents had the same nicotine concentration and that the results of the assay were not influenced by variation in food material. Midguts of these larvae were dissected so that the food content of the midgut remained intact. Ends of each midgut were sealed with clamps and the sealed midgut was carefully submerged in 500µL of bathing buffer [NaPO4 pH 7.0 and 0.3M sorbitol] in a 30mm petri plate. Midguts that were punctured during the dissection or had lost food material were not used. A 50µL aliquot of bath-buffer was collected at 0, 5, 10, 15, 20, 30, and 60 min (for control and CYP-silenced larvae, for each time-interval, n= 5). Nicotine concentrations of these collected bath-buffer samples were measured by HPLC/ESI-Q3-MS (Varian 1200).

Volatile nicotine trapping

Volatile nicotine was measured by adsorbing it on the pieces (2mm of PDMS tubing (Reichelt Chemietechnik, Germany).

Measuring nicotine in larval headspace

Nicotine in larval headspace was trapped for 1h in a sealed and ventilated glass vial (5cc), having a PDMS tube suspended in the headspace from the seal with the help of a solid needle (Fig. S5*B* and *SI Materials and Methods*).

Headspace-nicotine during the no-choice assays with perfuming

Nicotine in the container of no-choice assays with perfuming was trapped for 1h on the PDMS tube suspended in the chamber; adsorbed nicotine was extracted and quantified using HPLC/ESI-Q3-MS (Varian 1200). (*SI Materials and Methods*).

Trapping nicotine emitted from spiracle and cuticle

To evaluate if the levels of nicotine exhaled from larval spiracles varied with respect to hemolymph nicotine concentration, different amounts of nicotine (0.001, 0.002, and 0.004% of FM) were injected into the hemolymph of AD-fed larvae (7.0± 0.25g FM). Injection volume was kept constant (50µL) for every larva and the dorsal point between 5th and 6th body segments was used as the injection site (*SI Materials and Methods*). To measure nicotine emitted from spiracles, a PDMS tube was glued around the spiracle without disturbing its opening movement, using instant-adhesive (Fig. 4*B*). PDMS tubes were attached around the spiracle for 2h, after which they were carefully detached for nicotine extraction. A similar procedure was used to trap nicotine emitted from the spiracles of nicotine-injected (0.002% of FM) control and CYP-silenced larvae. However, to trap the spiracle-emitted nicotine ingested by the control and CYP-silenced larvae (which had fed on EV or irCYP plants, respectively), PDMS tubes were attached around the spiracles of washed larvae. These larvae were fed on irPMT leaves for 2h prior to the attachment of the PDMS tubes.

To measure nicotine emitted from the cuticle of AD-fed larvae, PDMS tubes were attached to the dorsal tip of the cuticle. In each larva, only one spiracle was sampled; therefore the total amount of nicotine emitted by each larva was estimated by multiplying the amount of nicotine on one PDMS tube by 18 (total number of spiracles/ larva). Nicotine adsorbed on PDMS tubes attached to spiracle or cuticle was quantified using the internal standard (*SI Materials and Methods*).

Extraction and quantification of nicotine

Leaf and larval frass

Homogenized leaf material and larval frass were extracted in extraction buffer A (60% methanol containing 0.05% glacial acetic acid) and were chromatographed on Agilent-HPLC 1100 as described earlier (52); relative concentration of nicotine was estimated using the standard curve of external nicotine standards (*SI Materials and Methods*).

Hemolymph, bath-buffer and PDMS tube

2µL hemolymph or 50µL bath-buffer or the sampled piece of PDMS tube was mixed with 50µL extraction buffer B [60% methanol, 0.05% glacial acetic acid and 5ng d_3-nicotine (Cambridge isotope laboratories Inc, USA) as an internal standard]. These mixtures were centrifuged at 13.4g for 20 min, at 4°C. Clear supernatant was collected and analyzed using a HPLC/ESI-Q3-MS (Varian 1200) with 35V capillary voltage as described earlier (51).

Analysis of cotinine, CNO and NNO

The limits of detection for nicotine, cotinine, CNO and NNO were determined using U(H)PLC/ESI-qTOF-MS-. The efficiency of extraction of each compound from frass was determined by spiking varying amounts in the frass, extracting it and then quantifying it. . These compounds were then detected and quantified from the frass or hemolymph of control or CYP-silenced larval samples (*SI Materials and Methods*).

Statistical analyses

Significance ($P \leq 0.05$) of the binary results of all survivorship and predation assays was tested in contingency tables using Fisher's exact test (to facilitate the understanding of figures, these results are expressed in %). All the other quantitative data were subjected to one-way ANOVAs and the statistical significance ($P \leq 0.05$) was determined using Fisher's least significant difference *post hoc* tests.

Footnotes

[1]P.K. and S.S.P. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: baldwin@ice.mpg.de

Author contributions: P.K., S.S.P., A.S. and I.T.B. designed and performed experiments, analyzed data and wrote the manuscript.

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Supporting information

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Waldbauer assays for nicotine budgeting

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Volatile nicotine trapping

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Analysis of cotinine, CNO and NNO

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Table S3. Predation rates in no-choice assays with *G. pallens* and ant lions with *M. sexta* larvae fed on WT/EV, irPMT, or irCYP plants.

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Video S1. Spider's attack behavior when presented with EV- (A), ir CYP- (B) and irPMT-fed (C) *M. sexta* larvae.

Materials and Methods

Stem-feeding nicotine to *N. attenuata* leaves

Completely expanded mature rosette stage *N. attenuata* leaves were detached from the plant along with their petioles. Through the perforated lid of a Teflon tube (15cc), the petiole was immersed in either a 1mM nicotine or water (control) solution, as shown in Fig. S1*D*. These tubes were incubated for 24h at 26°C/16h light, 24°C/ 8h dark and 60% humidity in a growth chamber (Snijders Scientific). After 24h of stem-feeding, leaves were harvested for the analysis of nicotine contents (Fig. S1*E* and *F*) or fed to *M. sexta* larvae using HPLC (Agilent 1100 series).

RNA isolation and quantitative real time PCR

Persistence of CYP6B46 silencing during nicotine flux determination experiments.

In the nicotine flux determination experiments, to render the larvae nicotine-free or to feed them the same diet as that of control larvae, 4th instar CYP-silenced larvae were fed (for 6-12h) on irPMT or EV plants, respectively. To evaluate if the CYP silencing persisted in such CYP-silenced larvae when they were feeding on other hostplants not expressing dsRNA of *Ms*CYP6B46, CYP6B46 transcripts were profiled in their midguts after larvae had fed on irPMT or EV plants for 24h (Fig. S4*B*).

Waldbauer assays for nicotine budgeting

Freshly hatched *M. sexta* neonates were placed on EV, irCYP and irPMT rosette-stage plant leaves and larvae were allowed to feed until they had reached the 4th instar. The mass of each larva was recorded. All larvae were then starved for 4h to empty their guts. Again after starvation, the mass of each larva was measured. Each larva was then provided with a known mass of leaf material from the same genotype of plant that they had been fed previously. Larvae were allowed to feed for 24h in an incubator maintained at 26°C/ 16h light, 24°C/ 8h

dark and 60% relative humidity. Blotting paper disks of known masses were placed at the bottom of the assay container to absorb any excreted liquids. After 24h of feeding, the mass of each larva and the mass of remaining leaf material were recorded. All the larvae were again subjected to 4h of starvation, during which time they had emptied their guts. Frass excreted by each larva, during the 24h of feeding and the 4h of starvation were collected, weighed along with paper disk and stored at -80°C until further use. Mass lost by the leaves of each line, due to evaporation in the incubator during the 24h assay was recorded and used to correct wet-to-dry mass conversion values. Nicotine levels of fresh and weight-loss leaves of each line were measured by HPLC (Agilent 1100 series) (see 'Extraction and quantification of nicotine'). The mass lost by the leaves of each line in the 24h feeding trial was used to calculate the exact amount of leaf material not consumed by each larva after 24h; this allowed for the quantification of actual amount of nicotine ingested by each larva. Nicotine levels in the collected frass samples (along with the blotting paper discs) were also measured and corrected considering the mass of the blotting paper disk. Percentage of nicotine excreted was determined by calculating the ratio of amount of nicotine excreted/ amount of nicotine in the ingested food X 100.

Volatile nicotine trapping

In all the experiments, before measuring volatile nicotine, larvae were washed with water at least three times, in order to remove any potential background signal of exoskeleton-adsorbed nicotine arising from direct larval contact with the food. 1mL water was used for each wash of 2nd instar larvae and 20mL water was used for 4th instar larvae. Larvae were washed until the nicotine concentration of the wash reduced below the detectable limit of HPLC/ESI-Q3-MS (Varian 1200) (see 'Extraction and quantification of nicotine').

Measuring nicotine in larval headspace

Washed larvae were placed in a sealed glass vial (5cc) fitted with a PDMS tube suspended in the headspace from the seal with a solid needle; an injection needle (0.08X40mm, BRAUN, Germany) was inserted in the seal for ventilation (Fig. S5*B*). Each larva was incubated in this setup for 1h at 30^{o}C, before extracting nicotine from the PDMS tube (see 'Extraction and quantification of nicotine').

A standard curve based on the amounts of PDMS-adsorbed nicotine was analyzed to evaluate the linear response of PDMS to increasing nicotine concentrations. We incubated 3, 6, 12, 25, 50, 100 or 200 ng nicotine in 5µL methanolic solution in the vial in volatile trapping setup (n=4; 4 replicates per nicotine concentration), for 1h. Nicotine adsorbed on the PDMS

tube was extracted and measured by HPLC/ESI-Q3-MS (Varian 1200). A standard curve was used to evaluate the linearity of adsorption of volatile nicotine onto PDMS; the response was linear (R^2= 0.98) over a 0-200ng of volatilized nicotine (Fig. S5*C*). To evaluate how much of the nicotine present in the trapping vial had the potential to be volatilized and therefore could be adsorbed to the PDMS tube, 100ng nicotine (n= 4) was allowed to evaporate for 1h. After incubation, 60± 6% (mean± SE) nicotine was found to be adsorbed on the PDMS tube and 34± 3% (mean± SE) remained in the vial, suggesting that almost all the volatilized nicotine was adsorbed on the PDMS tube. Therefore in the choice and no-choice predation assays, the amount of nicotine recovered from the PDMS tubes proportion was considered to be the same as that emitted by the larvae.

To quantify the nicotine emitted by the spider's larval prey, we evaluated how much of the volatile nicotine (present in the vial) was adsorbed on the PDMS tube. PDMS tubes were individually exposed to 100ng nicotine (n= 4) for 1h. After incubation, nicotine adsorbed on the PDMS tube was extracted and measured by HPLC/ESI-Q3-MS (Varian 1200). Nicotine that did not volatilize and remained in the vial after the 1h assay was also collected and quantified; to measure the amount of nicotine lost during the collection of the non-volatilized nicotine remaining in the vial, we extracted vials that had been spiked with 100ng nicotine immediately after the nicotine was applied (allowing the least time for volatilization) and quantified the nicotine. Since all the applied nicotine could be recovered from this rapid collection, the efficiency of collection was considered 100%. The amount of nicotine missing from the collection after 1h incubation was considered to be volatilized; the proportion of this missing nicotine that was actually recovered from PDMS tube was calculated and this proportion was used to calculate the quantity of nicotine emitted by the 2nd instar larva (used in the choice and no-choice predation assays).

Headspace-nicotine during the no-choice assays with perfuming

A PDMS tube was suspended in each no-choice assay container from the seal attached to a solid needle, immediately after placing the larva and the cotton swab used to perfume the chamber. Volatile nicotine in the headspace of larva used in water- or nicotine-perfumed no-choice assay was allowed to adsorb on the PDMS tube for 1h. Adsorbed nicotine was extracted and quantified by HPLC/ESI-Q3-MS (Varian 1200) (see 'Extraction and quantification of nicotine'); since these quantifications were relative, the values were used only for comparing the headspace nicotine content of the water- and nicotine-perfumed larvae.

Trapping nicotine emitted from spiracle and cuticle

Larvae that bled after injection were not used in the analysis. The injection site was carefully cleaned, the wipes were extracted and the nicotine concentration of these wipes was determined using HPLC/ESI-Q3-MS (Varian 1200) (see 'Extraction and quantification of nicotine'). Larvae which had leaked some of the injected nicotine onto the cuticle around the injection site were also not included in the analysis. Any potential differences in nicotine emission from the different larval spiracles were randomized amongst treatments by randomly selecting a different sampling spiracle in every biological replicate. Sampling-location bias was likewise avoided by randomly selecting a different sampling body segment, in every biological replicate.

Not all the nicotine emitted by the sampled spiracle was adsorbed to the PDMS tube and nicotine lost to the environment could not be quantified. Therefore the results obtained from this analysis were only used to compare the relative emissions of control and CYP-silenced larvae.

Extraction and quantification of nicotine

Leaf and larval frass

1mL nicotine extraction buffer A (60% methanol containing 0.05% glacial acetic acid) was added to 100mg crushed leaf or to 50mg crushed frass in a 2mL Eppendorf tube. Samples containing extraction buffer were homogenized using ceramic beads (0.9 g: Sili GmbH, Germany) on Geno/Grinder 2000 (Elvatech, Ukraine) for 2min with 600 strokes/min. Homogenized samples were centrifuged at 13.4 g for 20min, at RT. Supernatant was transferred to a fresh 1.5mL Eppendorf tube and was centrifuged again at 13.4g for 20min at 4°C. Clear supernatant was collected and analyzed on HPLC (Agilent 1100 series) as described by Keinaenen *et al* (42).

Analysis of cotinine, CNO and NNO

Cotinine was procured from Sigma-Aldrich (Germany). Cotinine N-oxide (CNO) was synthesized as described by Dagne and Castagnoli (1) and nicotine 1-N-oxide (NNO) was synthesized as described by Craig and Purushothaman (2). Retention times and molecular ions of these compounds were determined by injecting 1ng of each of these compounds onto a Phenomenex Gemini NX 5 (5 x 2.0 mm) U(H)PLC column (particle size 3 µM) with solvent A (0.1% ammonium hydroxide in ultrapure Millipore H_2O, pH 10) and solvent B (100% methanol). The gradient of 0 min/ 5% B, 0.5min/ 5% B, 2 min/80% B, 6.5min/80% B,

8.5min/5% B, 10min/ 5% B was used. Compounds were detected using a qToF-mass spectrometer (micrOTOF QII Bruker Daltonik, Bremen, Germany) equipped with an electrospray ionization (ESI) source in positive ion mode (instrument settings: capillary voltage, 4500V; capillary exit, 130V; dry gas temperature, 200C; dry gas flow, 8L/min). Calibration was performed using sodium formate clusters (10 mM solution of NaOH in 50/50% v/v isopropanol/water containing 0.2% formic acid).

0.05, 0.25, 0.5, 1.0, 2.0 or 4.0ng of each above-mentioned compound (3 replicates/ each concentration of compound) was analyzed with a system. For each compound, the lowest amount (among these injected quantities) that was detected by the micrOToF MS was considered to be the limit of detection.

The efficiency of extraction of each compound from frass was determined as follows. Crushed dried frass (50mg) of irPMT fed larvae was spiked with 50, 250, 500, 1000, 2000 or 4000ng nicotine, cotinine, CNO or NNO (3 replicates/ each concentration of compound). Spiked frass samples were extracted in 1mL extraction buffer C [60% methanol containing 0.05% glacial acetic acid and 1µg each of d_3-nicotine, d_3-cotinine, d_3-CNO and d_3-NNO (Cambridge Isotope Laboratories Inc, USA) as internal standards]. 1µL of these extracts were chromatographed and detected with a micrOToF mass spectrometer, as mentioned above. Detected quantity of the spiked compound was calculated relative to its respective d_3-internal standards from which the efficiency of recovery of each compound from frass was calculated. Efficiency of extraction from hemolymph was considered 100% for all the compounds.

To detect and quantify nicotine, cotinine, CNO and NNO in the frass or hemolymph of control or CYP-silenced larvae, 50mg frass or 50µL hemolymph was extracted in 1mL extraction buffer C. In addition, to be able to detect these nicotine metabolites if they were present in the frass at lower concentrations than that of nicotine, a part of each extract was concentrated 5-fold under vacuum. 1µL and 10µL of the original extracts and 10µL of the concentrated extracts were chromatographed, as described above.

Field predation assays and no-choice assays with *G. pallens* and ant lions

To evaluate the effect of dietary nicotine on *M. sexta*'s survival when exposed to the diurnal predators in the native habitat of *N. attenuata*, in 2004 we exposed day old, first instar larvae feeding irPMT or EV plants in the field, for 5h during the daytime. In 2012, survival of second-instar larvae fed on EV, irCYP or irPMT plants was assessed for 2d after placing on plants of the same genotypes in a predator-rich field plantation. During all the diurnal

survivorship assays, predation events by *G. pallens* were commonly observed. In no-choice assays, survival of second instar *M. sexta* larvae fed on WT, irPMT, or irCYP plants was determined when larvae were exposed to predation by ant lion larvae or *G. pallens* adults. Single larva was enclosed in soufflé cups (Solo 29.6mL, P100, Urbana, IL) with a moist paper tissue and a *G. pallens* individual and its survival was assessed after 1h (in 2012 and 2013 field seasons). One larva was dropped in each ant lion sand pit [pits in the field as well as the ones formed in soufflé cups (Solo 29.6mL, P100, Urbana, IL) by field collected ant lion larvae] and the immediate response (feeding or rejection) was recorded as described in Eisner *et al* (*33*) (in 2012 and 2013 field seasons); larval survival after 1h was also recorded (Table S3).

Supporting Figures

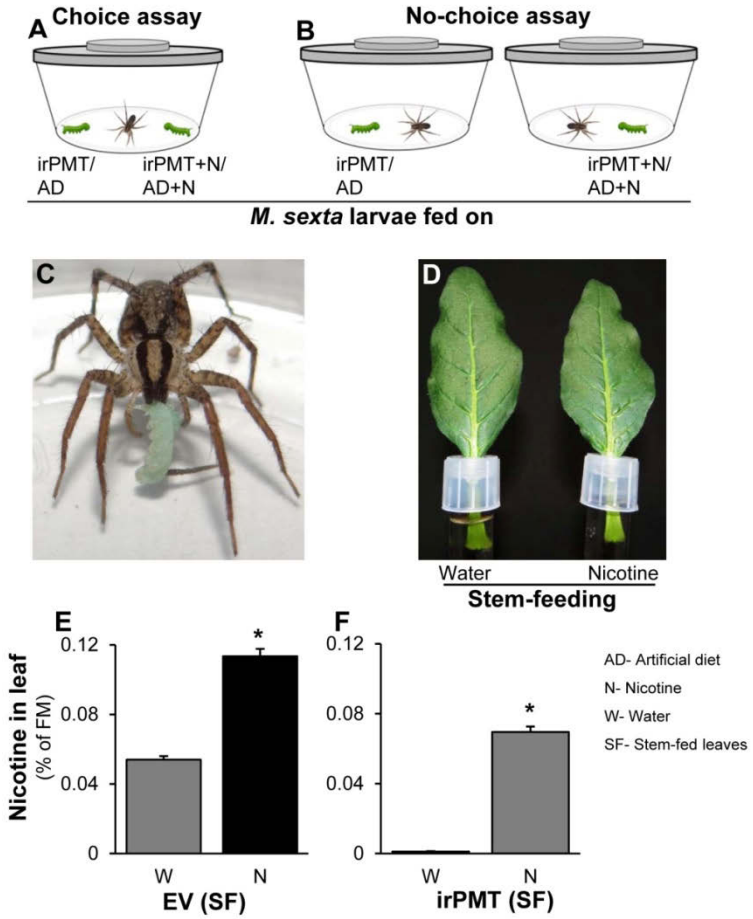

Fig S1. Setups for spider assays and stem-feeding nicotine into leaves and nicotine levels in control and stem-fed leaves

Schematic representation of the setup used for spider's **(*A*)** choice and **(*B*)** no-choice assays; both types of assays were conducted for 1h in 50mL polypropylene containers. **(*C*)** Spider feeding on 2nd instar *M. sexta* larva, during a no-choice assay. **(*D*)** Setup used to stem-feed leaves with water or 1mM nicotine. Nicotine levels (% of FM) of **(*E*)** EV ($F_{1, 16}$= 57.14, $P \leq 0.0001$, n=5) and **(*F*)** irPMT ($F_{1, 10}$= 444.45, $P \leq 0.0001$, n=6) leaves stem-fed water (W) or 1mM nicotine (N). Asterisks indicate significant differences determined by one way ANOVA. See Fig. 1 legend for the bar-shading codes.

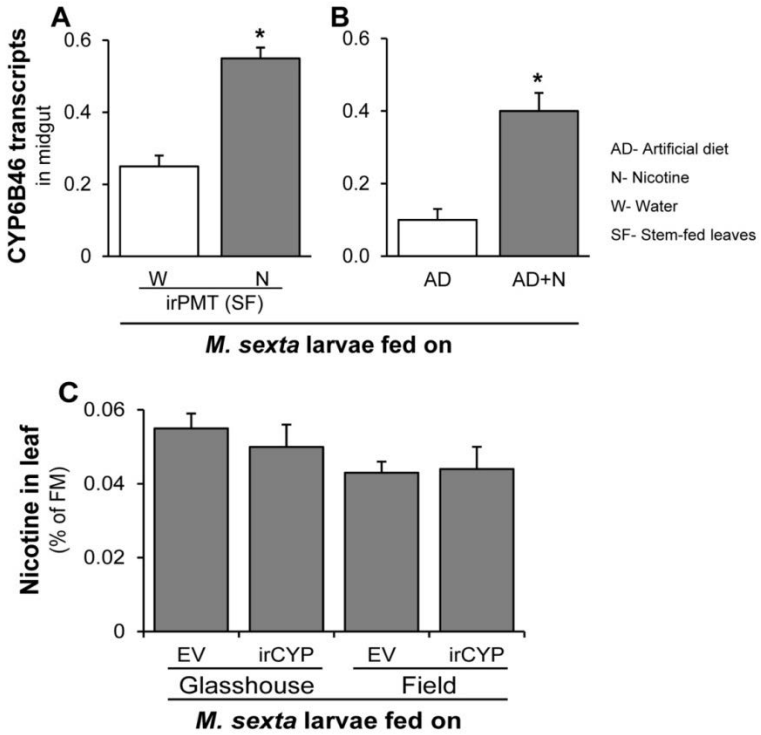

Fig S2. Midgut *Ms*CYP6B46 transcript levels in larvae feeding on *N. attenuata* leaves and artificial diets differing in nicotine contents

M. sexta CYP6B46 transcript levels (relative to ubiquitin) in midguts of 1st instar larvae fed (for 24h) on **(*A*)** W or N stem-fed irPMT leaves ($F_{1, 8}$= 116.25, $P \leq 0.0001$, n=5) and **(*B*)** AD and AD containing 0.1% of nicotine (AD+N) ($F_{1, 8}$= 8.10, $P \leq 0.05$, n=5). **(*C*)** Nicotine levels (% of FM) of the leaves of EV and irCYP plants grown in the glasshouse and the field (n=3). See Fig. 1 legend for the bar-shading codes.

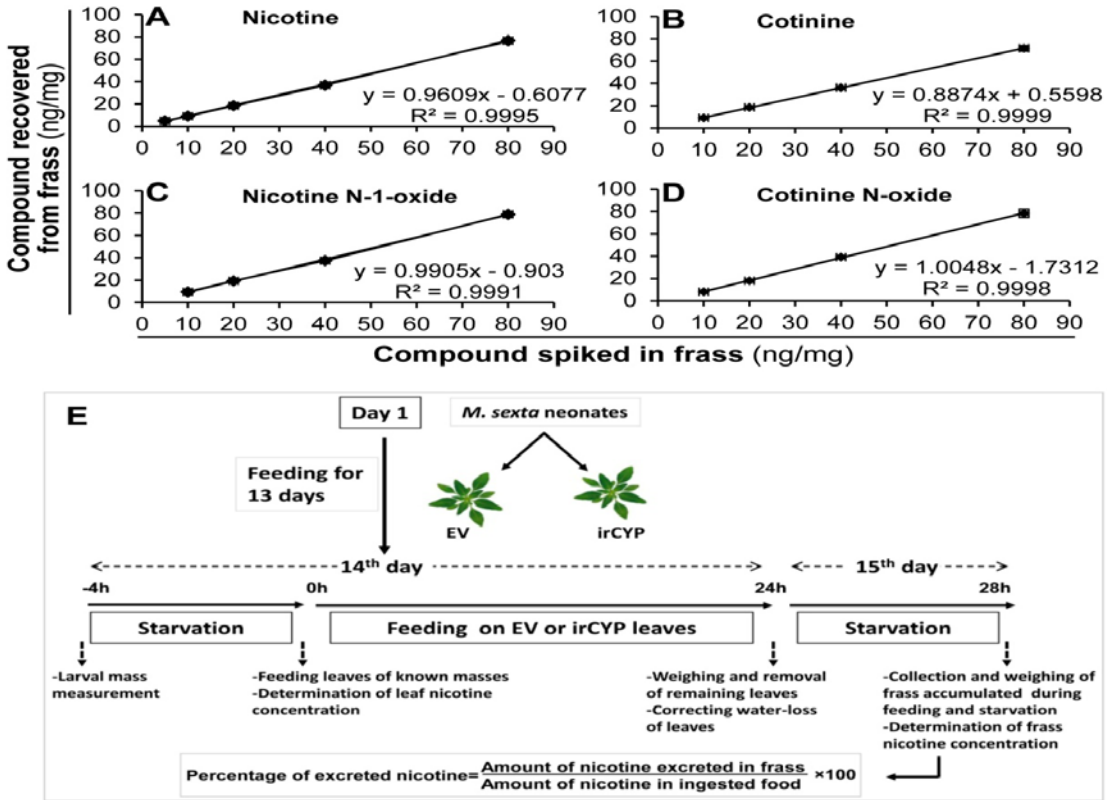

Fig S3. U(H)PLC/ ESI-QTOF-MS **based analysis of nicotine and its metabolites and the Waldbauer assay procedure**

Plots showing linear response of U(H)PLC/ ESI-QTOF-MS for **(*A*)** nicotine, **(*B*)** cotinine, **(*C*)** NNO and **(*D*)** CNO and the efficiency of their extraction (>90% for all the compounds) from standard addition experiments with frass; 50, 250, 500, 1000, 2000 or 4000ng of each compound was spiked in 50mg frass (n= 3 for each concentration of a compound). **(*E*)** Schematic of the Waldbauer assay used to quantify ingested and excreted nicotine and its metabolites in control and CYP-silenced *M. sexta* larvae.

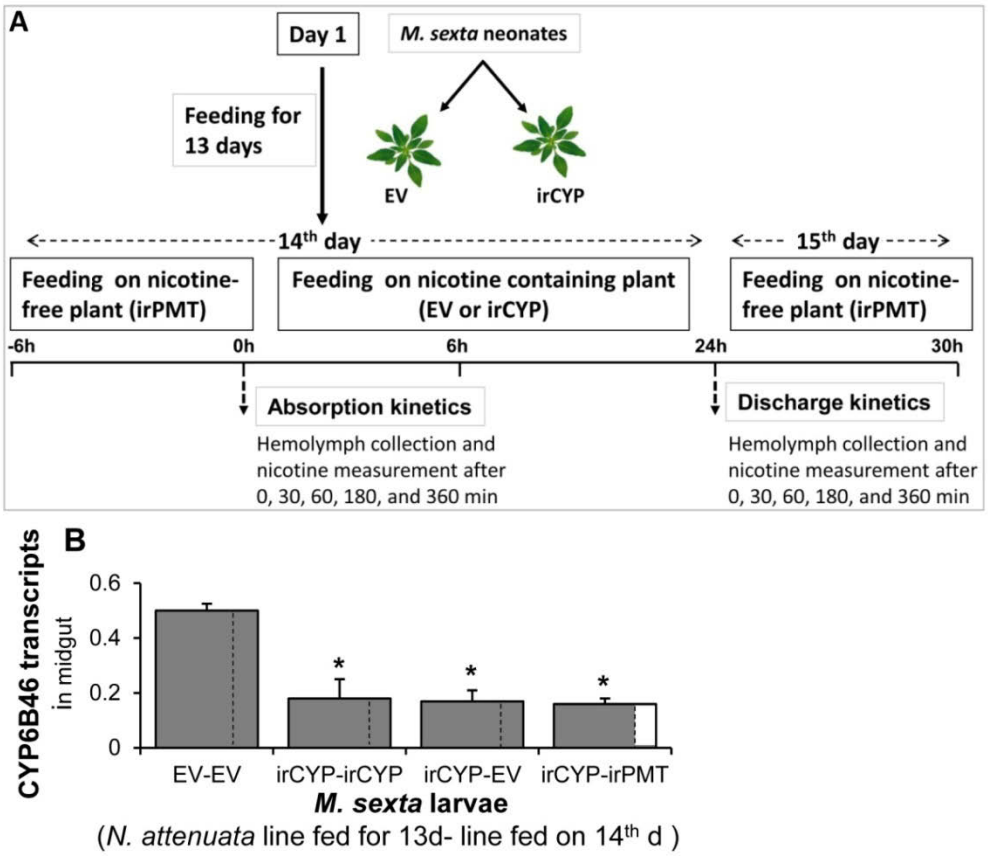

Fig S4. Schematic of the experiments used to determine the kinetics of nicotine flux in larvae and the persistence of CYP-silencing during this procedure

(*A*) Schematic detailing the experimental protocol used to measure the kinetics of nicotine absorption in and discharge from the hemolymph of control and CYP-silenced larvae. **(*B*)** CYP6B46 transcript levels (relative to ubiquitin) in midguts of 4th instar larvae that fed on EV or irCYP plants for 13d and then fed for 24h on EV, EV, irCYP or irPMT plants, respectively ($F_{3, 20}$= 164.28.2, $P \leq 0.0001$, n=5, 5, 8 and 6, respectively). Dashed line dividing each bar indicates the transfer on 13th day and the change in bar color indicates the relative nicotine concentration of the diet. See Fig. 1 legend for the bar-shading codes.

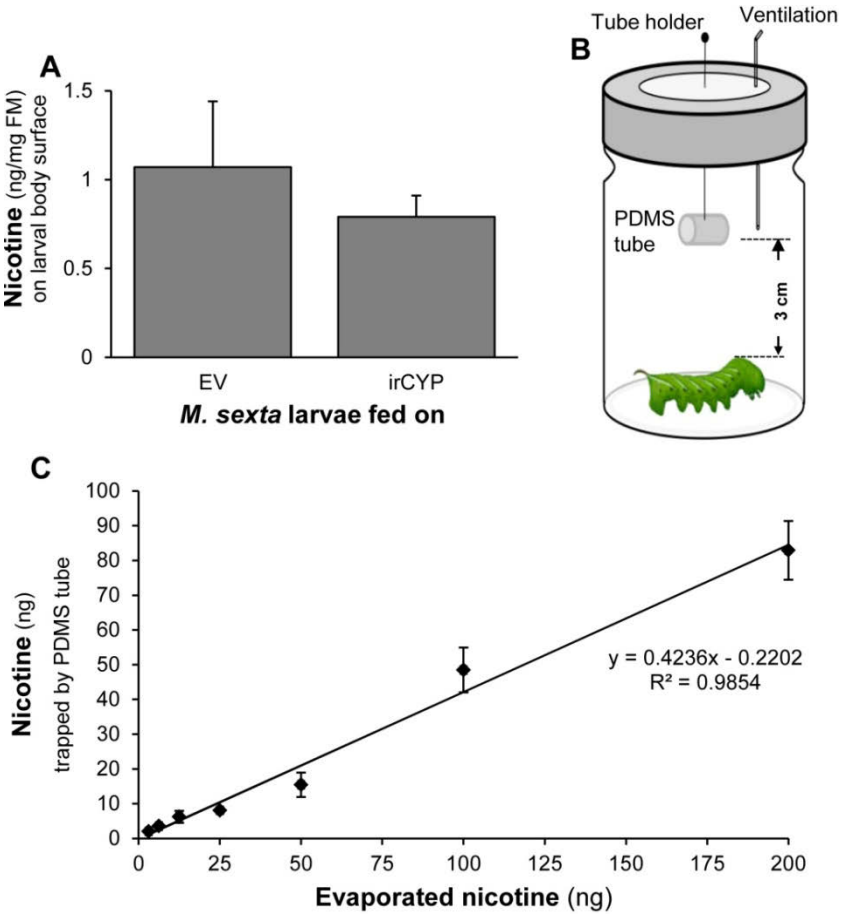

Fig S5. Trapping and quantification of nicotine from larval surface and in larval headspace

(*A*) Amount of nicotine (mean± SE) adsorbed to the body surface of control (n= 11) and CYP-silenced (n= 12) larvae; nicotine was recovered from the body-wash of intact larvae. **(*B*)** Schematic of the collection of larval nicotine headspace. **(*C*)** A standard curve based on the amounts of PDMS-adsorbed nicotine to evaluate the linear response of PDMS to the increasing headspace nicotine concentrations (n=4 for each nicotine concentration). See Fig. 1 legend for the bar-shading codes.

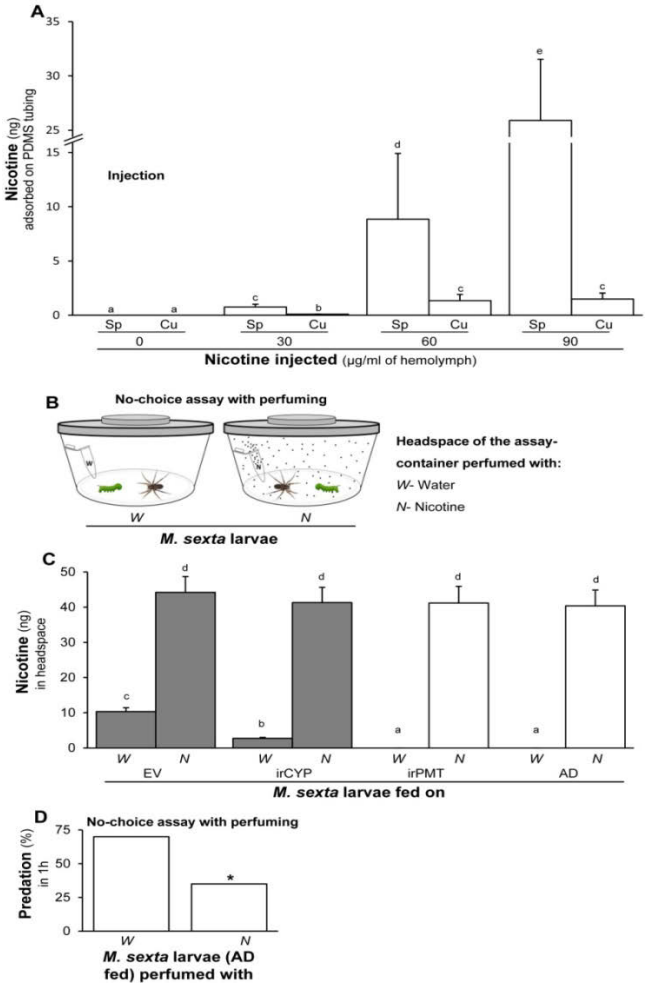

Fig S6. The release of nicotine through spiracles in larvae differing in hemolymph nicotine concentrations, schematic of no-choice assays with nicotine perfuming, nicotine in headspace of perfuming assays and consequences of perfuming on spider predation

(*A*) Relative amounts of nicotine adsorbed by the PDMS tubes attached to spiracles (Sp) and cuticle (Cu), after injecting differing amounts of nicotine into the hemolymph of artificial diet fed 4th instar larvae [(mean± SE) $F_{7, 40}$= 10.45, P≤0.0001, n=6]. **(*B*)** Schematic of a no-choice assay; assay environment was perfumed using 500µL of 1mM nicotine on a cotton swab (500µL water on a cotton swab was used as the control). **(*C*)** Relative amounts (ng) of nicotine (adsorbed by the PDMS tubes suspended) in the headspace of assay-containers of water- and nicotine- perfumed larvae, during no-choice assays [(mean± SE) $F_{7, 16}$= 42.01, P≤0.0001, n=3]; small letters indicate significant differences determined by one-way ANOVA. **(*D*)** Spider predation (%) on the larvae fed on AD after perfuming the (no-choice) assay environment with water (n=20) or nicotine (n=20); asterisk indicates significant difference (P≤0.05) by Fisher's exact test. See Fig. 1 legend for the bar-shading codes.

Tables

Table S1. Field survival of *M. sexta* larvae fed on WT/EV, irPMT, or irCYP plants. First-instar larvae pre-fed for 24h were exposed to native predators in a field plantation or native *N. attenuata* population, respectively. Survival recorded after 5h (2004) or 14h (2013) during daytime did not differ between WT and irPMT fed larvae; it also did not differ when recorded after 2d (2012). However, survival of irPMT and irCYP fed larvae was significantly lower than that of WT/EV fed larvae during night times (2013). *P*-values refer to Fishers exact test.

Year	Assay no.	N	Assay duration	% Survival			*P*
				WT/EV	irPMT	irCYP	
2004		15	5h	66.6	66.6	-	0.300
2012		13	2d	46.2	15.4	23.1	0.15 (irPMT) 0.21 (irCYP)
2013	Diurnal	50	14h	76	72	74	0.12 (irPMT and irCYP)
	Nocturnal	50	10h	80	50	50	0.045 (irPMT and irCYP)

* Daily replacement of dead larvae for the first 3 days

Table S2. Amounts of ingested and excreted food (leaf) and nicotine during the 24h Waldbauer assays. For a detailed description of the procedure of these assays, refer to Fig. S3*E* (n= 8; n.s.- no significant difference).

Parameter	EV fed larvae	irCYP fed larvae
Food (leaf) ingested (g)	3.5± 0.17	3.3± 0.38 (n.s)
Food excreted (g)	2.86± 0.67	2.64± 0.31 (n.s.)
Nicotine ingested (mg)	1.24± 0.2	1.09 ± 0.1 (n.s.)
Nicotine excreted (mg)	0.5± 0.1	0.63± 0.05 (*P*=0.01)
% nicotine excreted (of ingested)	40.11± 8.9	59.98± 5.18 (*P*=0.01)

Table S3. Predation rates in no-choice assays with *G. pallens* and ant lions of *M. sexta* larvae fed on WT/EV, irPMT, or irCYP plants. First instar larvae were dropped in native ant lion pits and second instar larvae were used in predation assays in cups with both predators

Predator	Year	N	% Larvae predated		
			EV	irPMT	irCYP
Ant lion	2012	26	70.0	70.0	70.0
	2013	15	73.3	73.3	66.6
G. pallens	2012	10	70.0	60.0	70.0
	2013	15	66.6	66.6	60.0

Table S4. APHIS notification numbers under which transgenic *N. attenuata* seeds were imported and plants released at the field station in Utah.

Line	Import #	Year	Release #
EV	07-341-101n	2012	11-350-101r
		2013	12-333-101r
irPMT (*Na*PMT NCBI accession no. AF280402)	04-020-07n	2004	04-020-08n
	07-341-101n	2012	11-350-101r
		2013	12-333-101r
irCYP (*Ms*CYP NCBI accession no. GU731529)	10-004-105m	2012	11-350-101r
		2013	12-333-101r

Videos

Video S1. Spider's attack behavior when presented with EV- (A), CYP- (B) and irPMT-fed (C) *M. sexta* larvae.

A spider in the assay container (50cc) was offered *M. sexta* larvae (2nd instar) that had fed since hatching on (A) EV, (B) irCYP, or (C) irPMT plants. Video shows how the spider is rapidly repelled by larvae fed on EV plants after a first contact, but readily attack and consume larvae had fed on the nicotine-free irPMT plants or the nicotine-replete irCYP plants that had silenced their midgut expressed *Ms*CYP6B46 transcripts.

Supporting references

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Chapter 5

Manuscript III

Difference in nicotine metabolism of two herbivores of *Nicotiana attenuata* renders them differentially susceptible to a common native predator

MANUSCRIPT III

Difference in nicotine metabolism of two herbivores of *Nicotiana attenuata* renders them differentially susceptible to a common native predator

Pavan Kumar, Preeti Rathi, Matthias Schoettner, Ian T. Baldwin and Sagar S. Pandit

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Abstract

In the native habitat, *Nicotiana attenuata* hosts a niche membered by several herbivores and their natural enemies. It produces nicotine to protect itself from the herbivores. However, *Manduca sexta*, the specialist herbivore of *N. attenuata*, tolerates this toxin. Mechanisms behind *M. sexta*'s exceptional nicotine tolerance remained debated over the years. Mainly, two theories have been proposed. The first one states that larvae oxidize nicotine to less toxic nicotine N-oxide (NNO), cotinine and cotinine N-oxide (CNO) using cytochrome P450s (CYPs); being polar, these oxides are thought to be amenable to faster excretion than that of nicotine. The second theory rules out the oxidation and suggests that nicotine is rapidly excreted by the larvae, well before it is metabolized. Congruent to this theory, we recently discovered that *M. sexta* larvae do not oxidize nicotine. Instead, they keep nicotine unmetabolized and externalize it for deterring the nicotine-sensitive predatory spiders. Thus *M. sexta* larvae are benefitted by the unmetabolized nicotine. Here we tested if indeed the oxides are less toxic to *M. sexta* than nicotine and if larvae excrete them faster than nicotine. None of the oxides were found to be less toxic than nicotine and all were excreted at the same rate as that of nicotine. Next, we analyzed the other herbivores of *N. attenuata* namely *M. quinquemaculata*, *Spodoptera exigua*, *S. littoralis* and *Heliothis virescens*. We found that *S. exigua*, *S. littoralis* and *H. virescens* oxidize nicotine whereas *M. quinquemaculata* does not. Cotinine and cotinine-N-oxide (CNO) could be found in the hemolymph and frass, whereas nicotine N-oxide (NNO) was found only in the frass of nicotine oxidizing larvae. We used *S. exigua* as a nicotine oxidizing model to test if the nicotine oxides confer lower toxicity than nicotine to the larvae that synthesize these oxides; we fed these oxides to larvae through the diet and measured the larval mass and mortality. To these

larvae nicotine was the most detrimental, whereas NNO was the least, suggesting that NNO is not only an oxidation product but it could also be a detoxification product. Further, we also studied the ecological benefits of nicotine-oxides to *M. sexta* and *S. exigua*. We tested the deterrence ability of nicotine oxides against the nicotine-sensitive wolf spiders. Nicotine or nicotine oxide fed or coated *M. sexta* and *S. exigua* larvae were offered to the spiders and their predation events were measured. None of the nicotine oxides deterred spiders. Only nicotine fed or coated larvae of both the species deterred spiders. Unlike *M. sexta* in which the predation on nicotine fed and coated larvae was similar, in *S. exigua*, although not significantly, nicotine fed larvae were predated more than the nicotine coated larvae. Moreover, when the spiders were asked to choose between the nicotine fed *M. sexta* or *S. exigua* larvae, it preferred *S. exigua*. The reason for this bias could be that the oxidation might have left lower amount of unmetabolized nicotine for externalization in this larvae. We verified this by complementing nicotine externally to *S. exigua* larvae by coating it on their body surface or by perfuming the assay environment; in both these cases spider preference was found to be diminished and the predation rate reduced to the level of that on nicotine fed *M. sexta* larvae. We infer that oxidation of nicotine by *S. exigua* could be due to a general spontaneous response to the ingested xenobiotic and all the oxides may not confer the detoxification effect. In fact, possession of nicotine oxides renders them susceptible to the spiders than the possession of unmetabolized nicotine. On the contrary, *M. sexta* is ecologically benefitted by keeping nicotine unmetabolized. Since *S. exigua* lowers its nicotine content by oxidation, it may provide a gateway for the nicotine-sensitive members of higher trophic levels in *N. attenuata*-hosted niche. Our results also suggest that host allelochemistry and selection pressure from predators could have been two of many factors behind the specialization of *M. sexta* on nicotine containing plants.

Introduction

Terrestrial ecosystems or niches comprising of plant as a primary food and energy source, insect herbivores as primary consumers and their natural enemies (predators and parasites) as secondary consumers have been extensively studied for the infochemical networks that drive the interactions between the trophic levels of these systems. This

infochemistry is highly dynamic. Insect herbivores locate their hosts using the volatile-bouquets emitted by them (Bartlet *et al.* 1993). Herbivores of different feeding guilds or frequently even of same feeding guilds induce different defense responses in the same host (Voelckel & Baldwin 2004; Vogel *et al.* 2007; Govind *et al.* 2010). On the other hand, some herbivores are more tolerant to their hosts' defenses than the others (Cornell & Hawkins 2003; Wittstock *et al.* 2003; Ahn *et al.* 2011). In the systems involving more than one host plant, herbivores fed on certain hosts render less palatable to the their natural enemies than those fed on the other hosts (Dyer 1995).

In the Great Basin Desert of Utah (USA), *Nicotiana attenuata* (Solanaceae) hosts one such niche that involves various lepidopteran, hemipteran and orthopteran herbivores and their natural enemies like ants, lizards, preying mantids, bugs and beetles (Fig. 1). The most frequent and most defoliating herbivores of *N. attenuata* are lepidopterans (Fig. 1). *Manduca sexta* (Sphingidae) is a chief herbivore, which is also considered to be a specialist herbivore of this plant; the next major herbivore is *Spodoptera exigua* (Noctuidae) which is also considered to be a generalist, since it feeds on the plants from many other families (Fig. 1) (Steppuhn & Baldwin 2007). Apart from these, *M. quinquemaculata* (Sphingidae) and *Heliothis virescens* (Noctuidae) also occur on *N. attenuata*; the former is considered as a specialist whereas the later as generalist. Against these herbivores, *N. attenuata* produces various direct defense chemicals; nicotine, diterpene glycosides and proteinase inhibitors are produced upon herbivory so they are called induced defenses, whereas compounds like chlorogenic acid and rutin are constitutively present. Among these, nicotine is the major defense and is influential against most of the herbivores (Steppuhn *et al.* 2004). In tobacco leaves it occurs constitutively (0.1–1% of the dry mass) and is also induced upon a herbivore attack (up to 4% of the dry mass) to reduce herbivore performance and fertility (Gordon 1961; Baldwin 1988; Krug & Proksch 1993; Ohnmeiss *et al.* 1997; Steppuhn *et al.* 2004; Lambers *et al.* 2008) and allows plants to prevent the generalist herbivore from compensating for plant protease inhibitors (Steppuhn & Baldwin 2007).

M. sexta is known to be the most nicotine-tolerant herbivore (Wink & Theile 2002). In fact, it has been used as a model to study nicotine toxicity and tolerance in

insects. Nevertheless, the studies on its nicotine metabolism have yielded contradictory results (Appel & Martin 1992) based on which, two major theories have been proposed. According to the first theory, nicotine is detoxified by oxidation. The major pitfall of this theory is that the nicotine oxidation products reported in different studies were not similar; Snyder et al (1993 and 1994) reported cotinine and cotinine N-oxide (CNO), Wink & Theile (2002) suggested nicotine N-oxide (NNO), whereas unidentified conjugates of 5'-hydroxynicotine were also proposed as the major detoxification products (Morris 1983). Cytochrome P450s (CYPs) were thought to be responsible for such a oxidative detoxification (Snyder *et al.* 1993; Snyder *et al.* 1995; Wink & Theile 2002) nevertheless, none of the studies provided the evidence on specific CYP genes mediating the formation of nicotine oxides, in *M. sexta* larvae. The second theory suggested that nicotine is rapidly excreted via Malpighian tubules before its modification takes place (Self *et al.* 1964a; Maddrell & Gardiner 1976). In our recent work, similar to Maddrell & Gardiner (1976) and self et al (1964a and 1964b), we found that *M. sexta* larvae do not oxidize nicotine (Kumar *et al.* 2013). In fact, they benefit by not metabolizing it, as they transport the ingested nicotine from the midgut to spiracles via blood stream; from spiracles, it is exhaled in the headspace to deter the nicotine-sensitive wolf spiders [*Camptocosa parallela* (Lycosidae)]. In the native habitat, these spiders were found to be the major nocturnal predators of *M. sexta* larvae (Kumar *et al.* 2013).

Similar to several other xenobiotics, it is known that nicotine is not equally toxic to all the herbivores. The mass reduction and mortality in *M. sexta* larvae are much lower than the other frequent lepidopteran larvae such as *S. exigua*, when they feed on nicotine containing plants (Steppuhn & Baldwin 2007). Moreover, during herbivory, oral secretion of *M. sexta* larvae elicited the production of ethylene in the leaves that suppressed the expression of putrescine N-methyltransferase gene responsible for the biosynthesis of nicotine, whereas such suppression was not observed after the herbivory by *S. exigua* (Voelckel *et al.* 2001; Winz & Baldwin 2001; Steppuhn *et al.* 2004). Nicotine has also been found to regulate the interactions between herbivores and their natural enemies. Nicotine ingested by the herbivore exerted negative effects on its predators and parasitoids. On the other hand the parasitic wasps used nicotine as a cue to search the herbivore population (Barbosa *et al.* 1986).

Thus, within the *N. attenuata*-hosted niche, nicotine could be a central and important infochemical and deciphering its role is inevitable for understanding the infochemistry of this niche. To reveal nicotine's importance, we first reveal how differently the various herbivores of *N. attenuata* metabolize nicotine and what could be the physiological consequences of this metabolism. Ultimately, we test if the nicotine metabolites can also confer resistance against the native predators and if they are more influential than nicotine. This study also offers an opportunity to compare the generalist and specialist lepidopteran herbivores that co-occur in the niche of *N. attenuata* based on their nicotine metabolism and nicotine-mediated tritrophic interactions.

Results

Nicotine oxides are not formed by *M. Sexta* larvae

Although in our previous study the reported nicotine metabolites could not be detected in the laboratory reared and maintained *M. sexta* larvae, we hypothesized that (since the highly inbred *M. sexta* colonies are often used in the laboratory experiments) our previous results were obtained due to the usage of insects from the colony that originated from the oxidation-impaired parent (s); thus nicotine-oxidation ability may still exist in the natural variants of *M. sexta* and similarly, in the natural variants of *M. quinquemaculata*. Variants of both these species did not show any of the reported nicotine oxides in hemolymph or frass (Table 1); moreover the nicotine levels in hemolymph and frass were similar in both the species [Hemolymph (µg/mL; mean± SE): *M. sexta*- 18.9± 1.29, (n=8); *M. quinquemaculata*- 11.93± 4.5 (n=6)] [Frass (ng/mg; mean± SE): *M. sexta*- 427.35± 37.36 (n=8) *M. quinquemaculata*- 358.85± 45.67; (n=6)]. This supported our previous results and advocated the absence of nicotine-oxidation strategy in *M. sexta* and *M. quinquemaculata*.

Waldbauer assay based budgeting of nicotine and nicotine oxides was conducted, considering the hypothesis that *M. sexta* larvae lost the early step of nicotine oxidation. We fed cotinine and NNO to analyze CNO. We also fed CNO to test whether CNO is completely utilized by larvae. No significant difference in the excreted amount of nicotine or nicotine oxides (% of total ingested) was found, by Waldbauer assay (Fig.

2A). In addition no CNO was detected in the hemolymph and frass of cotinine and NNO fed larvae. Chromatograms and U(H)PLC/ESI-QTOF MS mass spectra of nicotine, NNO, cotinine and CNO analyzed was compared with deuterated forms of these metabolites to confirm their identity (Supplementary figure 1A- D). U(H)PLC/ESI-QTOF MS responses to the nicotine and nicotine oxides was investigated by standard curves of nicotine, NNO, cotinine and CNO along with their the deuterated forms. U(H)PLC/ESI-QTOF MS responses to all the metabolites were linier suggesting our analytical techniques were reliable (Supplementary figure 2A- D). In addition, extraction procedure efficiency tested by measuring percent recoveries of nicotine, NNO, cotinine and CNO from frass. Extraction efficiency of all the metabolites were found to be equal (80%) and there was no degradation over 24h in the frass also confirmed (Supplementary figure 3A). Further we studied, if the components of AD itself cause chemical oxidation of nicotine also tested by mixing nicotine with AD, incubating for 24h (0h incubated samples were used as controls), extraction and analysis using U(H)PLC/ESI-QTOF MS. None of the oxides were detected from the AD diet extracts, indicating no auto-oxidation of nicotine.

To compare the clearance rates of nicotine and nicotine oxides from larval hemolymph, we introduced them directly to larval hemolymph by injection and so avoided their entry in the digestive track; we periodically measured their concentration in hemolymph until 6h, when all the metabolites attained the steady purge rate and hemolymph was almost clear of them. Clearance rates of injected nicotine and nicotine oxides from hemolymph were found to be similar (Fig. 2B)

To study the induction response of previously reported CYPs, which are induced in response to nicotine, transcript abundance of CYP6B46, CYP4M1 and CYP4M3, were measured in midguts of *M. sexta* larvae fed AD containing nicotine and nicotine oxides (Snyder *et al.* 1993; Govind *et al.* 2010; Kumar *et al.* 2013). Nicotine as well as nicotine oxides was found to induce CYP6B46 (Fig. 2C). Even after this induction the further oxidized products of NNO, cotinine or CNO were not found in the hemolymph or frass of the larvae that fed on these metabolites. CYP4M1 and CYP4M3 were not induced in the larval midguts by any of the ingested metabolites CYP4M3 (Supplementary figure 3B

and C). Lastly, nicotine oxidation is not *M. sexta*'s strategy is supported by the phenotype that cotinine feeding caused melanism in *M. sexta* larvae (Fig. 1D).

Noctuid herbivores oxidize nicotine

Generalist and specialist herbivores perceive the host plant defense metabolite differently (Ali & Agrawal 2012). We studied the nicotine detoxification strategy of generalist insect herbivores. Qualitative analysis of hemolymph and frass of *N. attenuata* fed *S. exigua*, *S. littoralis* and *H. virescens* showed that all of them oxidize nicotine (Table 1). Quantitative analysis and comparison of nicotine metabolites in the hemolymph and frass of *S. exigua* larvae suggests that cotinine and CNO were the major nicotine metabolites formed. Nicotine was the only metabolite found in the headspace of these larvae (Fig. 3A- C). Similar nicotine oxidation pattern seen when *S. exigua* larvae fed *N. attenuata* foliage (Supplementary figure 4A- C). No nicotine oxides found in hemolymph, frass and headspace samples of *M. sexta* larvae.

Among the tested compounds, cotinine is more toxic to *M. sexta* and nicotine to *S. exigua*

To test whether nicotine oxides are detoxified products, performance of nicotine oxides fed *M. sexta* and *S. exigua* larvae measured. *M. sexta* larvae feeding AD containing nicotine oxides (NNO and cotinine) was not better than that of larvae feeding on nicotine. In fact, mass of larvae feeding AD containing cotinine was significantly lower than that of nicotine feeding larvae (Fig. 4A). Performance of *S. exigua* larvae feeding AD containing cotinine and CNO was similar to that of larvae feeding AD containing nicotine, whereas the performance of NNO feeding larvae was better (Fig. 4B). Altogether performance data indicates nicotine was more lethal to *S. exigua* larvae whereas cotinine was more lethal to *M. sexta* larvae, among all the metabolites tested (Fig. 4C).

Only the externalized nicotine confers spider-deterring ability to *M. sexta* and *S. exigua* larvae and nicotine oxides do not

Recently we demonstrated that *M. sexta* larvae avoid oxidation of nicotine and emit ingested nicotine to deter spider (Kumar *et al.* 2013). Spider-deterring ability of nicotine

oxides tested by no-choice spider predation assays using *M. sexta* and *S. exigua* larvae. Spiders are not deterred by the NNO, cotinine or CNO fed/ coated *M. sexta* or *S. exigua* larvae (Fig 5A and B). However, nicotine coated *S. exigua* larvae are 2-fold more spider-deterrent than the nicotine fed *S. exigua* larvae. By spider-choice assay we found spiders prefer nicotine fed *S. exigua* larvae over nicotine fed *M. sexta* larvae (Fig. 5C), since *S. exigua* oxidizes nicotine. This preference is diminished when nicotine is topically applied to the larvae (Fig. 5D) or when nicotine is perfumed in the assay containers (Fig. 5E). Altogether spider predation data indicates deterrence ability is associated with nicotine and not nicotine oxides and larvae oxidizing nicotine become susceptible to spider attack compared to non-oxidizing larvae.

Major diurnal predator *G. pallens* is also not deterrent by nicotine oxides

Earlier, we demonstrated that another abundant predator, the *G. pallens* was not deterred by the nicotine feeding larvae (Kumar *et al.* 2013). However, it is plausible that the herbivores co-opt unmetabolized xenobiotics against one predator and the metabolized ones against another. Therefore, we tested if various nicotine oxides confer the herbivores an advantage against *G. pallens* that has a diurnal preying time (as against the nocturnal spiders) and a different prey selection and feeding behavior than the spider. *G. pallens* was not deterred by the NNO, cotinine and CNO fed (Supplementary figure 5A) or coated (Supplementary figure 5B) *M. sexta* and *S. exigua larvae*

Discussion

Nicotine oxidation is not responsible for *M. sexta*'s nicotine tolerance

Plant allelochemicals not only influence insects' growth and reproduction but also their susceptibility to predators and parasitoids. Often herbivores adapt to the host plant allelochemicals either by means of detoxification, sequestration or rapid excretion. Our recent work demonstrated that *M. sexta* does not oxidize nicotine and it acquires spider-resistance by exhaling unmetabolized nicotine. Here, we tested if natural variants of *M. sexta* and *M. quinquemaculata*- the close relative of *M. sexta*, oxidize nicotine; however, nicotine oxides could not be detected in the in hemolymph and frass of both the species,

suggesting that oxidation of nicotine is not nicotine tolerance strategy of these specialist herbivores. Previous results showing nicotine oxidation in *M. sexta* could be ascribed to the influence of experimental design and methods. Snyder et al attributed their success of CNO detection to the highly advanced HPLC technology of their time and predicted that the use of technologies such as LC-MS-MS would provide further clarity (Snyder *et al.* 1994). We did not detect any nicotine oxide even though we used the further advanced U(H)PLC/ESI-QTOF-MS technology. We used fourth instar larvae in contrast to the fifth instar (which might differ physiologically from the previous four growth-focused instars as they are a preparatory stage for pupation) that were used by Snyder et al (1994) as well as by Wink and Theile (2002). Similar to Self et al (1964a), we always fed the larvae on physiologically realistic concentrations of dietary nicotine (up to 0.1% of diet FM, levels found in *N. attenuata* leaves), whereas Snyder et al (1994) and Wink and Theile (2002) used the AD reared fifth instar larvae, which were never exposed to nicotine during their previous instars; these larvae were then abruptly exposed to high and physiologically unrealistic amounts of nicotine (0.75 and 1.0% of fresh diet, respectively). It should also be noted that we always used freshly collected frass and hemolymph, whereas previous workers used the stored and dried frass (Snyder *et al.* 1994). Lastly, like us, the possibility that nicotine could be oxidized by the chemical components of diet (before it is ingested) was never tested, so it is also possible that the oxides seen by the previous researchers were formed by the AD's reaction with nicotine.

Snyder et al (1994 and 1995) proposed that CNO was the final product of nicotine oxidation pathway; it could be formed either through cotinine or NNO intermediate. Each step is an oxidation mediated by a CYP. Since we could not detect any nicotine oxide in *M. sexta* and *Ms*CYP4M1 and *Ms*CYP4M3 that were presumed to be responsible for nicotine oxidation were not induced upon nicotine ingestion, we hypothesized that in *M. sexta* individuals that we used, one or more oxidation steps could be inactive or absent (Snyder *et al.* 1993; Snyder *et al.* 1995). So if the pathway's intermediate step's substrate is supplied, *M. sexta* might be able to process it to the final product of the pathway. Therefore, we fed nicotine oxides (separately) to *M. sexta* larvae and tried to find the further oxidized product or the final product in their hemolymph and frass. We also conducted Waldbauer assay based budgeting of these fed compounds considering that if

one of these products was used up by *M. sexta* in its primary metabolism, it will be found in lesser concentration in frass than the other metabolites (Waldbauer 1982). However, the Waldbauer assay results showed that the excretion of none of the fed compounds was notably disproportionate (Fig. 2A). The other possibility was that these oxides were purged from the hemolymph faster than nicotine and such a temporally disproportionate purge could not be detected by Waldbauer assays because they are based on the collective excretion of 24h. Hence clearance rates of each metabolite from hemolymph tested. The clearance of all the metabolites (including nicotine) from hemolymph took place at a similar rate falsifying the long standing hypothesis that being more polar than nicotine, oxides are amenable to faster excretion than that of nicotine (Snyder *et al.* 1994).

M. sexta larvae use pumps to purge toxic alkaloids and protect the nervous system from them; these pumps function against most of the structurally similar alkaloids like nicotine, morphine, atropine or even alkaline synthetic dyes (Maddrell & Gardiner 1976; Murray *et al.* 1994). Earlier we demonstrated that *Ms*CYP6B46 is a part of such pump (Kumar *et al.* 2013). Our Waldbauer assay results in combination with these previous results also indicated that 'purge by excretion' could be a strategy of *M. sexta* against all the structurally similar alkaloids and coherent to this work- the nicotine oxides. Indeed, *Ms*CYP6B46 was found to be induced by all the nicotine oxides. But even after this induction the further oxidized products of nicotine, NNO or cotinine were not found in the hemolymph or frass of larvae that fed on these metabolites; this added to the evidence on the absence of nicotine oxidation mechanism in *M. sexta* and strengthened the theory on *Ms*CYP6B46 being a component of alkaloid pump.

N. attenuata's noctuid herbivores oxidize nicotine

Host plant defense adaption strategies of various insect herbivores are often different (Govind *et al.* 2010; Ali & Agrawal 2012). In the *N. attenuata*-hosted multitrophic niche, where nicotine is a major metabolite of the host that reaches almost every trophic level, such variation in nicotine metabolism strategies of the primary consumer can restrict the flow of nicotine in the niche and enable the survival nicotine-sensitive components in this nicotine controlled milieu. We analyzed if the other lepidopteran herbivores also keep nicotine unmetabolized like *M. sexta*, or as previously reported for some insects (Self *et*

al. 1964b), they oxidize nicotine to form NNO, cotinine and CNO. Indeed, all the noctuid herbivores (*S. exigua*, *S. littoralis* and *H. virescens*) were found to lower the nicotine content by oxidizing it to NNO, cotinine and CNO. We selected *S. exigua* as nicotine oxidizing herbivore model to study the consequences of oxidation. When nicotine cotinine and CNO were fed through AD, mass of *S. exigua* larvae reduced to the half of that of AD fed larvae; mass of NNO fed larvae remained intermediate. The effect of nicotine oxides was similar to that observed on *M. sexta* larvae except that cotinine caused severe mass reduction in *M. sexta*. Interestingly, nicotine caused significantly high mortality in *S. exigua*, whereas in *M. sexta* such high mortality was caused by cotinine.

Nicotine oxidizing herbivores render susceptible to the predators

We further tested if this variation in nicotine metabolism of two major herbivores can be perceived by the natural enemies at the next trophic levels. Predation of spiders over the nicotine and nicotine oxides fed or coated larvae revealed that oxidized forms of nicotine were not deterrent to the nicotine-sensitive spiders (Kumar *et al.* 2013). In these assays only nicotine fed or coated *M. sexta* and *S. exigua* larvae significantly deterred spiders. Given the choice, among the nicotine fed *M. sexta* and *S. exigua* larvae, spiders chose the later. We hypothesized that such bias in spider behavior was caused due to oxidation, *S. exigua* lost the unmetabolized nicotine that could be externalized against the spiders or could be too low to volatilize. This was proved to be true when we could diminish the spiders' bias with the *S. exigua* larvae were complemented with nicotine- either by coating on their body surface or by perfuming the assay environment with nicotine, as described by Kumar et al (Kumar *et al.* 2013). Equal non-responsiveness of *G. pallens* to nicotine and its oxides supported the line that both nicotine-sensitive and nicotine-insensitive branches exist in this niche. Together, these results suggested that *S. exigua* being more sensitive to nicotine, adopts the detoxification strategy that causes a reduction in nicotine flux towards the next trophic levels; this behavior may provide a gateway for the nicotine-sensitive predators to this niche. On the other hand, *M. sexta* manages to co-opt nicotine to keep the nicotine-sensitive predators away.

In the native habitat, the sand beds in which *N. attenuata* grows, the pits formed by ant lions frequently occur. These are interesting predators in *N. attenuata*'s niche, as they

keep waiting in their pits for the prey to fall into it. We have previously shown that ant lions are insensitive to nicotine (Kumar *et al.* 2013). *S exigua* larvae feeding on *N. attenuata* plants drop down to the ground more frequently than *M. sexta* larvae. So these larvae are more prone to the predation by ant lions. Since these larvae lower their nicotine content and produce nicotine oxides, the effect of nicotine oxides on ant lions would be worth to study in future.

Significance to the generalist-specialist paradigm

The generalist-specialist paradigm has always been intriguing to biologists. In case of herbivores, parameters like host range, ability to manipulate the host and its defense, tolerance to host's allelochemicals, earning the refuge from the host against natural enemies and co-opting the host defenses against them must be considered before designating them as generalists or specialists (Ali & Agrawal 2012). However, the 'specialist' or 'generalist' designations are generously used for convenience; previous studies have often been criticized for using inappropriate controls, comparing the insects of two different feeding guilds, small sample size and for conducting a plant- or insect-biased study (Bernays & Graham 1988; Dyer 1995; Ali & Agrawal 2012). Since a comprehensive investigation may never be possible using a single system, the critics have always asked for sufficient empirical evidences from assorted systems, for the realization of this paradigm. In this work, we attempted to differentiate generalists and specialists (as they were pre-designated) based on their physiological response to the major xenobiotic of their host plant. We tested if they differ in metabolizing the host's defense xenobiotic and in using it against their natural enemies. We kept the least possible variability in the test conditions of two herbivores. Although they belong to different lepidopteran families, we analyzed *M. sexta* as specialist and *S. exigua* as generalist, since they have the same feeding guild and they have often been regarded and compared as Solanaceae-specialist and -generalist, respectively (Steppuhn *et al.* 2004; Voelckel & Baldwin 2004). In fact, the oxidative response against the xenobiotics that *S. exigua* and other noctuids showed against nicotine has been described as a characteristic of generalist herbivores (Krieger *et al.* 1971; Ahmad 1983). Here, we studied the generalist-specialist paradigm in a meticulous manner by exposing the generalist and specialist (having the same feeding guild) to the common milieu that included the same host plant (*N. attenuata*), same plant

defense xenobiotic, nicotine and the common native predators. Based on the general alkaloid responsive induction behavior of CYP6B46 in *M. sexta* (even after which the alkaloid oxidation was not observed), it can be postulated that like generalist, the oxidative detoxification systems in specialist herbivore are also a subject to general induction by various xenobiotics; this can also be seen as a remnant of generalist response that these species lost on the way to specialization. It appears that specialists *M. sexta* and *M. quinquemaculata* benefit at the ecological level by not detoxifying nicotine, whereas the generalists oxidize nicotine (probably, to gain the physiological comfort), at the cost of their survival in the spider-rich native habitat.

The notion that herbivore specialization is an evolutionary advance is quite loosely supported; especially because the term 'specialization' is largely generalized, especially when the herbivore's specialization is defined only in terms of the narrowness of its host range. Ali and Agrawal (2012) pointed that the specialization should be seen in light of varied selection traits such as diet breadth, mechanism of host identification, degree of tolerance to hosts' defense metabolites, capability to induce favorable response in the host and to co-opt host-defenses against their natural enemies. Bernays and Graham (1988) stated that there is ample evidence that urge for resource availability and nutrition could have stemmed the food plant flexibility in generalists; however the selection pressures behind the evolution of specialist behavior were unclear, although plant allelochemistry and chemical coevolution of herbivores were thought to be the most acceptable explanations. It is evident that along with food and energy, *N. attenuata* also produces nicotine that is spread in the niche via herbivores. Our results substantiate the notion that plant allelochemistry exerts a bottom-up selection force on herbivores and highlight the importance of host chemistry in the long-debated generalist-specialist paradigm; they also support that the natural enemies are significant selective forces in the evolution of herbivore-host plant relationship and that they constitute the top-down selection force.

In nut shell, *M. sexta*'s exceptional nicotine tolerance cannot be attributed to the oxidative detoxification. In fact, nicotine oxidation is harmful to *M. sexta* if the products like cotinine are formed. Nicotine oxides are not excreted faster than nicotine by *M. sexta*. It retains the ecological advantage against the nicotine-sensitive predatory spiders

by keeping nicotine unmetabolized. Since nicotine oxides are not deterrent to the spiders, generalist noctuid *S. exigua* larvae that form these oxides render susceptible to the spiders. Thus nicotine could be the bottom-up and spiders could be the top-down selection force in *N. attenuata*-hosted nicotine-controlled niche.

Materials and methods

Plant material

Nicotiana attenuata 30x inbred seeds, which were originally collected in 1988 from a native population at Utah, USA were used. Seeds were germinated on sterile Gamborg B5 medium (Duchefa, Harleem The Netherlands) after 1h of treatment with diluted smoke (House of Herbs, Passaic, NJ) and 1µM GA_3 (Roth, Karlsruhe Germany) Ten days after germination, seedlings were transferred into Teku pots containing a peat-based substrate, and after an additional 10 to 12d, the plantlets were transplanted into individual 1L pots with the same substrate (Krügel *et al.* 2002). In the glasshouse, plants were grown at 24°C to 26°C, relative humidity approximately 60%, and supplemented with light from 400- and 600-W sodium lamps (Philips, Herrsching Germany) for 16h (Halitschke & Baldwin 2003).

Artificial diet (AD)

AD was used for the feeding experiments. AD was prepared and used according to Grosse-Wilde et al and Waldbauer (Waldbauer *et al.* 1964; Grosse-Wilde *et al.* 2011). AD was used to prepare the (0.1% FM) nicotine and nicotine oxides containing diets for feeding larvae.

Insects

M. sexta and *S. littoralis* eggs were used from the in-house reared colony (Grosse-Wilde *et al.* 2011). Eggs of *S. exigua* and *H. virescens* were ordered from Benzon Research Company (USA). *M. quinquemaculata* eggs were collected from *N. attenuata* or Datura plants at field plots (Great Basin desert Utah, USA). All the eggs were stored and larvae reared in a growth chamber (Snijders Scientific, Tilburg, Netherlands) at 26°C/ 16h light, 24°C/ 8h dark, until the larvae hatched. *M. sexta* and *M. quinquemaculata* neonates were individually transferred to 50 ml cups using forceps. Fresh AD was provided on every alternative day for all the insects. *S. littoralis* and *H.*

virescens neonates were initially reared on AD for 3- 4 days; later individual larva was transferred to solo cup, having a diet cake, using tiny painting brush.

Quantification of nicotine, NNO, cotinine and CNO from larval frass and headspace by HPLC and U(H)PLC/ESI-QTOF-MS

Analysis of nicotine and nicotine oxides were carried out as mentined by Kumar et al. Standard curves of nicotine and nicotine oxides were prepared to confirm the linier response of the U(H)PLC/ESI-QTOF-MS (Micro-TOF, Bruker- Germany). Efficiency of extraction of nicotine and nicotine metabolite from frass was estimated by spiking and re-extracting the metabolite, followed by measurement. Degradation of a metabolite during 24h assay or storage time between sample extraction and analysis was tested by spiking and recovery at 0 and 24 h respectively.

Feeding nicotine and nicotine oxides to *M. sexta* and *S. exigua* larvae

Nicotine and cotinine was procured from Sigma-Aldrich (Germany). Cotinine N-oxide (CNO) and nicotine 1-N-oxide (NNO) was synthesized in laboratory as reported previously (Kumar *et al.* 2013). 0.1% (FM) diet of nicotine, cotinine, NNO and CNO prepared by dissolving 100 mg of metabolite 500 µL of 100% MeOH and mixing later to 100gm of AD. Newly hatched larvae were transferred to to AD containing nicotine or nicotine oxides for rearing in growth chamber.

Nicotine and nicotine oxides coating on *M. sexta* and *S. exigua* larvae

AD fed *M. sexta* or *S. exigua* larvae under testing were dipped in 0.2% aqueous nicotine, NNO, cotinine or CNO separately and used for the assays.

Spider predation

C. parallela spiders were collected from in and around the *N. attenuata* field plantation (Great Basin desert Utah, USA) where they were particularly abundant. Spiders were placed individually in chambers and starved for 12h before all assays. Spider predation choice, no-choice and predation assays using second instar *M. sexta* and *S. exigua* larvae (fed 0.1% (FM) diet of nicotine, cotinine, NNO or CNO or dipped in

0.2% aqueous nicotine, NNO, cotinine or CNO separately) were performed according to Kumar et al (Kumar *et al.* 2013).

Geocoris predation no-choice assays

G. pallens predation no-choice assay were performed using *M. sexta* and *S. exigua* larvae fed 0.1% (FM) diet of nicotine, cotinine, NNO or CNO. AD fed *M. sexta* and *S. exigua* larvae dipped in 0.2% aqueous nicotine, NNO, cotinine or CNO separately were also used for the assays. *Geocoris* predation assays performed according to Kumar et al (Kumar *et al.* 2013).

Larval performance after feeding on nicotine oxides containing died

Freshly hatched (n=50) *M. sexta* and *S. exigua* neonates were transferred to AD alone or AD containing 0.1% (FM) of nicotine, cotinine, NNO or CNO. Larvae were reared on these diets for 10 days in insect growth chamber. Left over diet was replaced by fresh diet on daily basis. Number of larvae dead was also noted while replacing the diet. Larval mass measured on 10^{th} day.

Waldbauer assay

Waldbauer assays to budget the ingested and excreted nicotine and nicotine oxides by *M. sexta* larvae were performed. Freshly hatched 50 *M. sexta* neonates were transferred to AD alone or AD containing 0.1% (FM) of nicotine, cotinine, NNO or CNO. Larvae were reared on these diets for 13 d in insect growth chamber. On 14^{th} d Waldbauer assay was performed as reported earlier (Kumar *et al.* 2013).

Nicotine and nicotine oxides excretion kinetics

Freshly hatched *M. sexta* neonates were transferred to AD and reared for 14d in insect growth chamber. On 15^{th} day 100µL of water containing [0.001% (FM) of nicotine or nicotine oxide] was injected into the hemolymph of previously anesthetized (by exposing larvae to dry ice) larvae. Dorsal point between 5^{th} and 6^{th} body segments of larvae selected as injection point. All larvae used in this analysis were in the 4^{th} instar and of similar mass range (± 500mg). Hemolymph (2µL) of each larva was collected at 0,

30, 60, 180, and 360 min by clipping the tip of the larval horn and used for the analysis of injected metabolite by U(H)PLC/ESI-QTOF-MS(Kumar *et al.* 2013).

RNA isolation and real time quantitative PCR (qRT-PCR)

RNA isolation was performed using Trizol reagent (Invitrogen, Germany), according to the manufacturer's protocol. Quantitative real time PCR to measure CYP6B46, CYP4M1 and CYP4M3 transcripts level was performed as reported by Kumar et al (Kumar *et al.* 2012). Ubiquitin was used as an internal control to normalize the abundance of CYP6B46 transcripts.

Figures

Table 1. Qualitative profiles of nicotine metabolites in hemolymph and frass of various lepidopteran herbivores (for each species, n=5) of *N. attenuata*. Every species showed the same profile when fed on *N. attenuata* (WT) foliage or on AD containing 0.1% of nicotine. (NNO≡ nicotine N-oxide, CNO≡ cotinine N-oxide, H≡ Hemolymph, F≡ Frass, +≡ present and -≡ absent.)

Metabolite	***M. sexta***		***M. quinquemaculata***		***S. exigua***		***S. littoralis***		***H. virescens***	
	H	**F**	**H**	**F**	**H**	**F**	**H**	**F**	**H**	**F**
Nicotine	+	+	+	+	+	+	+	+	+	+
NNO	-	-	-	-	-	+	-	+	-	+
Cotinine	-	-	-	-	+	+	+	+	+	+
CNO	-	-	-	-	+	+	+	+	+	+

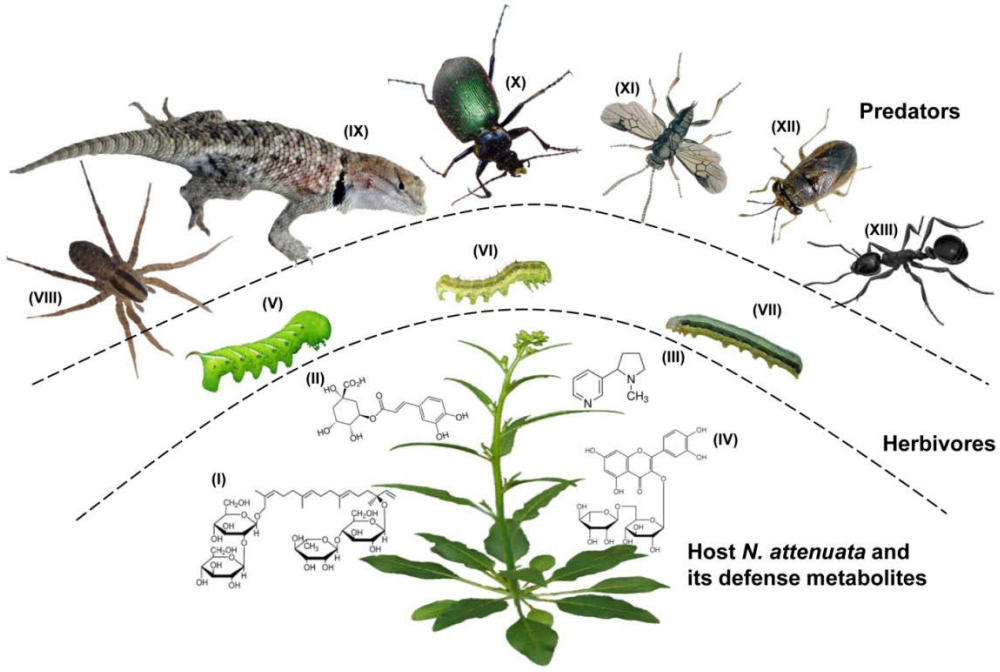


Fig. 1 The composition of *N. attenuata*-hosted niche
Host *N. attenuata*, its defense metabolites **(I)** diterpene glycoside **(II)** chlorogenic acid **(III)** nicotine and **(IV)** rutin, its major primary consumers, the lepidopteran herbivores **(v)** *M. sexta* **(VI)** *H. virescens* and **(VII)** *S. exigua* and the secondary consumers, the natural enemies of herbivores **(VIII)** wolf spider **(IX)** lizard **(X)** carabid beetle **(XI)** parasitic wasp **(XII)** big eyed bug and **(XIII)** ant.

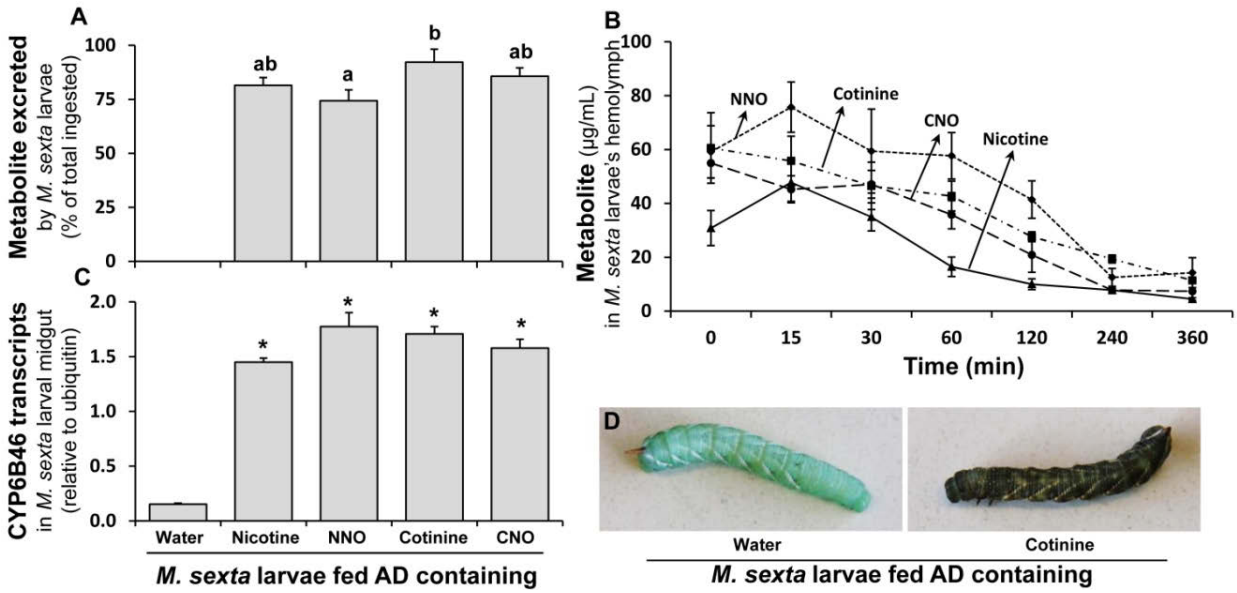


Fig. 2 Physiological consequences of nicotine oxides

(A) Waldbauer assay based quantification of excreted (% of total ingested) nicotine and nicotine oxides by 4th instar *M. sexta* larvae that were fed AD containing water (control) or 0.1% (FM) nicotine, NNO, cotinine or CNO [(mean± SE) F3, 22= 5.9, P≤0.05, n= 6 for nicotine, NNO and cotinine and 8 for CNO] **(B)** Discharge kinetics of nicotine and nicotine oxides from hemolymph of *M. sexta* larvae injected (into hemolymph) with 0.001% (of FM) nicotine, NNO, cotinine or CNO (n=5 larvae per metabolite) **(C)** CYP6B46 transcript levels (relative to ubiquitin) in midguts of 2d old 1st instar *M. sexta* larvae fed AD containing water (control) or 0.1% (FM) nicotine, NNO, cotinine or CNO [(mean± SE) F4, 19= 94.5, P≤0.05, n= 5] **(D)** Melanism in *M. sexta* larva fed AD containing 0.1% (FM) cotinine (right) [larva fed AD containing water (control) (left) was not melanized]. Small letters or asterisks above the bars indicate significant differences determined by one way ANOVA (P≤0.05).

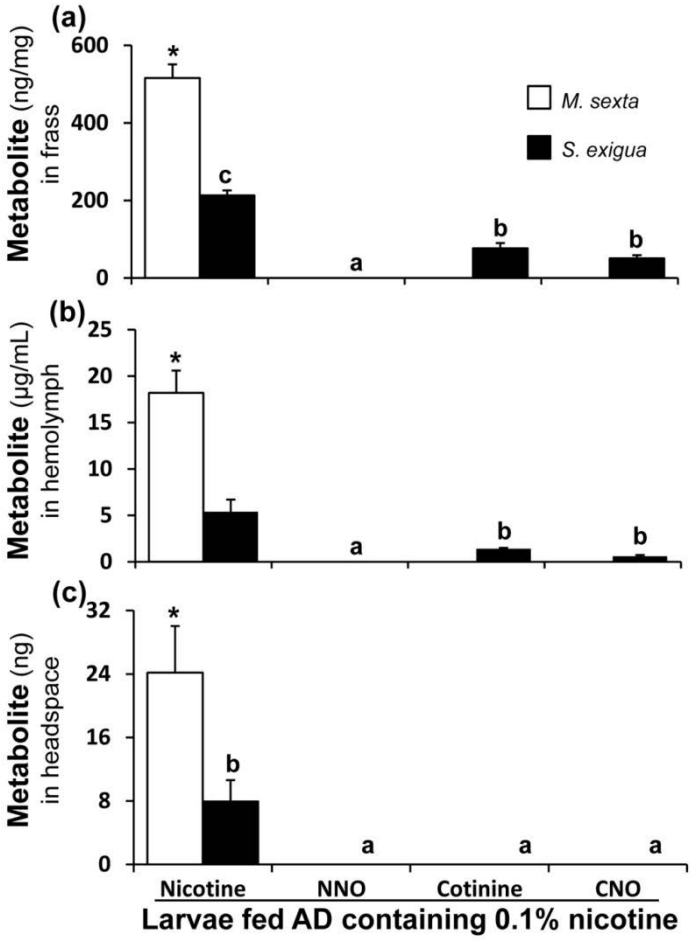


Fig. 3 Generalist herbivore *S. exigua* oxidizes nicotine, whereas specialist herbivore *M. sexta* does not

S. exigua oxidizes nicotine, but *M. sexta* does not. U(H)PLC/ESI-QTOF-MS-based quantitative analysis of nicotine, NNO, cotinine and CNO in **(a)** frass **(b)** hemolymph and **(c)** headspace of third-instar *M. sexta* (n= 5) and *S. exigua* (n= 5) larvae fed AD containing 0.1% (FM) nicotine. Lower-case letters above the *S. exigua* bars indicate significant differences ($P \leq 0.05$) among them, by one-way ANOVA. Asterisks above the *M. sexta* nicotine bars indicate that they differ significantly ($P \leq 0.05$) from the *S. exigua* nicotine values, as determined by one-way ANOVA. Nicotine oxides were not detected in *M. sexta*. The detection limit of nicotine was 0.25ng and 0.5ng for cotinine, CNO and NNO; efficiency of extraction was >90% for all these compounds.

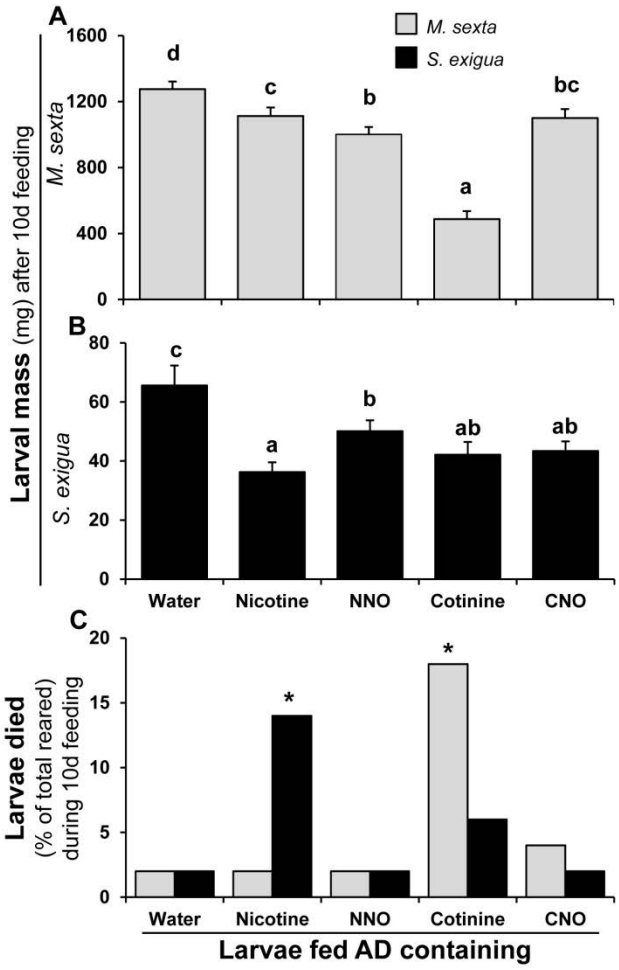


Fig. 4 Effect of nicotine and nicotine oxides on generalist and specialist herbivores Larval mass of (**A)** *M. sexta* [(mean± SE) F4, 172= 45.2, P≤0.05, n= 36, 34, 37, 33 and 37 for water, nicotine, NNO, cotinine and CNO, respectively] **(B)** *S. exigua* [(mean± SE) F4, 154= 6.03, P≤0.05, n= 32, 27, 37, 30 and 33 for water, nicotine, NNO, cotinine and CNO, respectively] and **(C)** percent of *M. sexta* and *S. exigua* larvae died after 10d feeding AD containing water (control) or 0.1% (FM) nicotine, NNO, cotinine or CNO; Every bar represents data from 30 larvae (n= 30). In A and B small letters above the bars indicate significant differences determined by one way ANOVA ($P \leq 0.05$); in C asterisks indicate significant differences ($P \leq 0.05$) by Fisher's exact test on frequencies (and not on the displayed percentages in the figures).

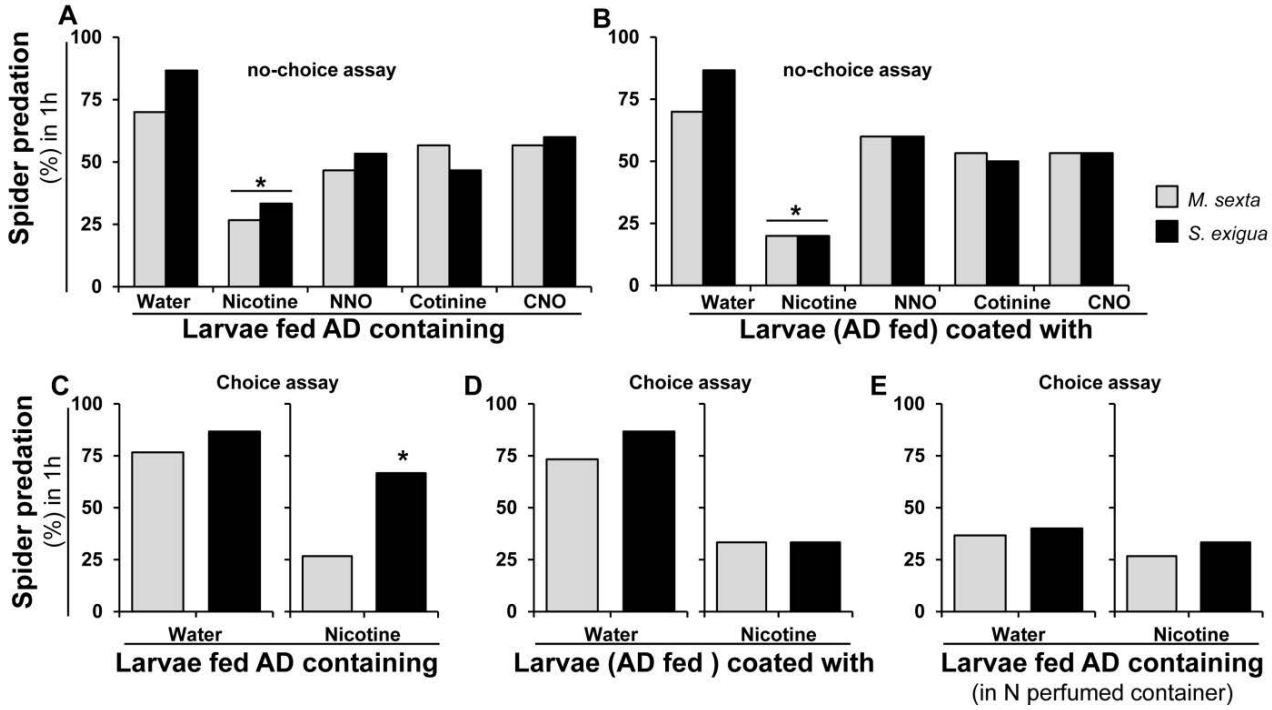
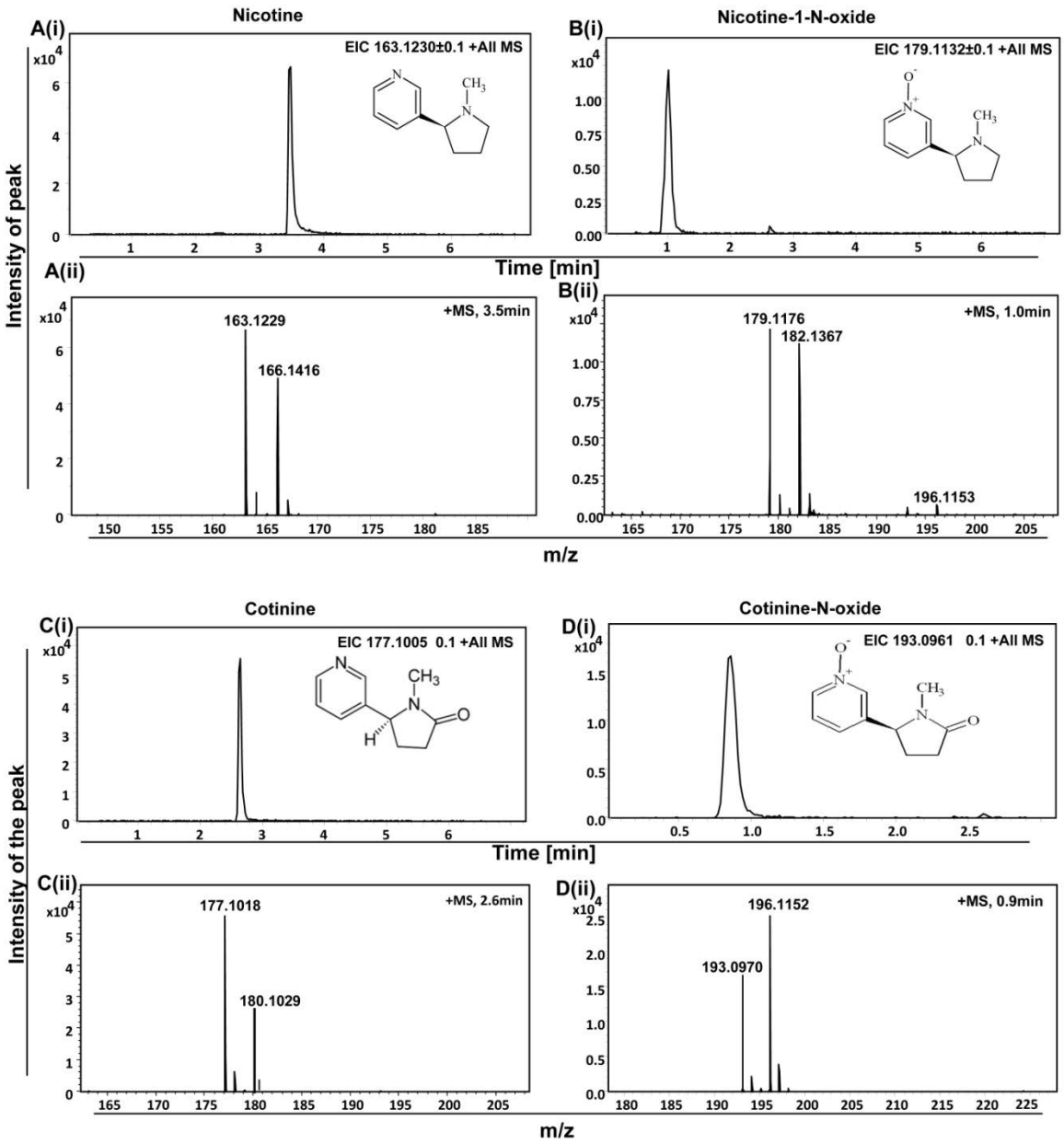
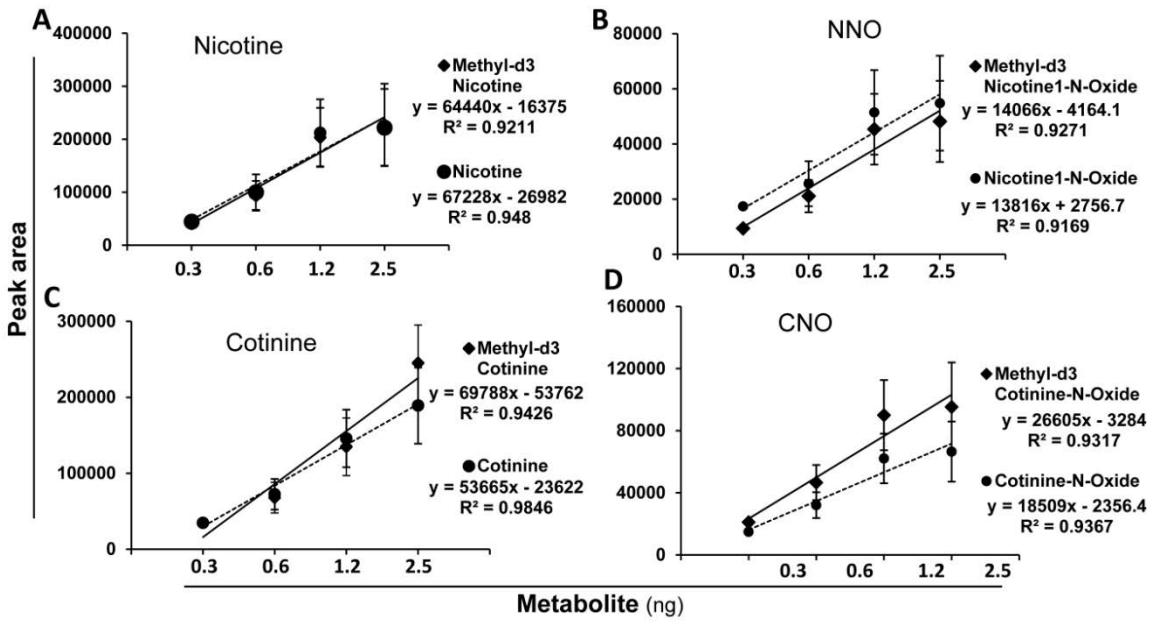


Fig. 5 Nicotine deters spider but nicotine oxides do not

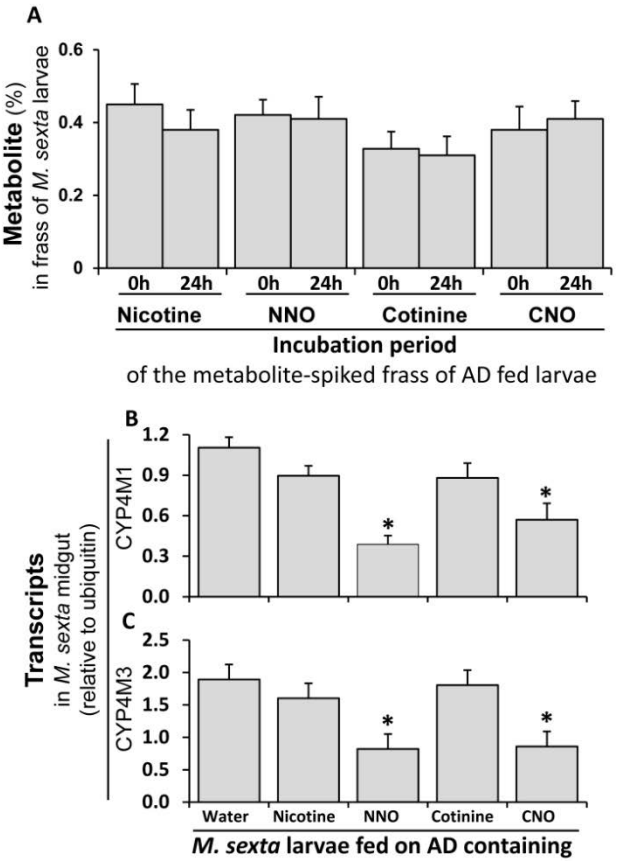
Spider predation (%) (in 1h no-choice assay) on 2nd instar *M. sexta* and *S. exigua* larvae **(A)** fed AD containing water (control) or 0.1% (FM) nicotine, NNO, cotinine or CNO and **(B)** fed AD and coated with water (control) or 0.2% aqueous nicotine, NNO, cotinine or CNO. Spider predation (%) (in 1h choice assay) on 2nd instar *M. sexta* and *S. exigua* larvae **(C)** fed AD or AD containing 0.1% (FM) nicotine **(D)** fed AD and coated with water (control) or nicotine **(E)** fed AD containing water (control) or 0.1% (FM) nicotine and having the assay environment nicotine-perfumed using 500µL of 1mM nicotine on a cotton swab. Every bar represents data from 30 larvae (n= 30). Asterisks indicate significant differences ($P \leq 0.05$) by Fisher's exact test on frequencies (and not on the displayed percentages in the figures).



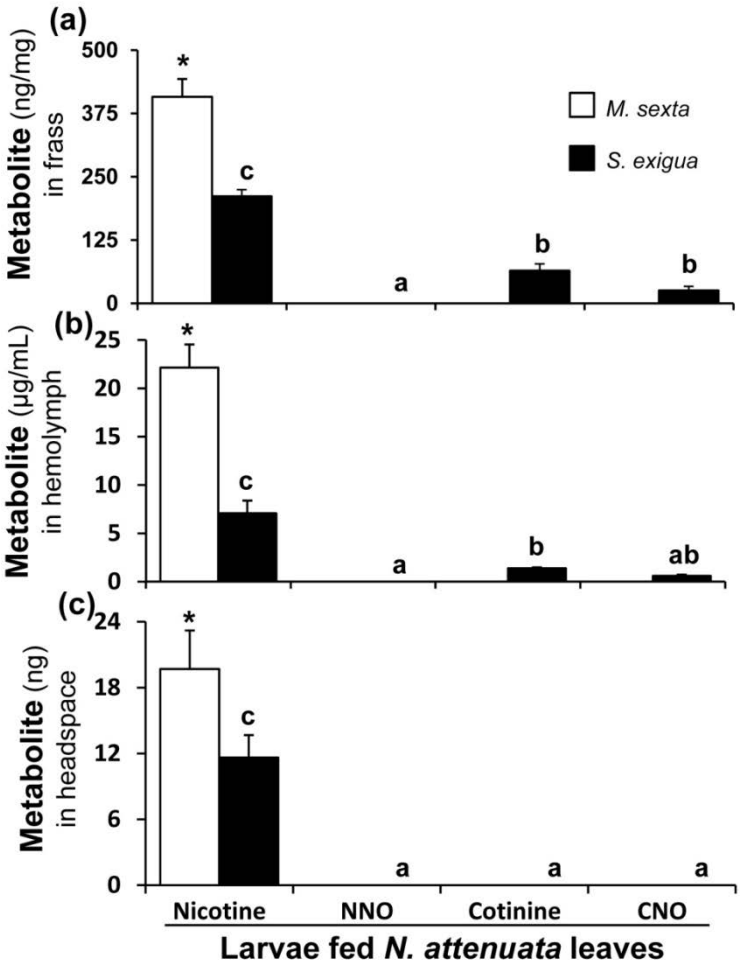
Supplementary Fig. 1 U(H)PLC/ESI-QTOF MS based analysis of **(A)** nicotine **(B)** NNO **(C)** cotinine and **(D)** CNO. **(i)** Extracted Ion Chromatograms (EICs) of metabolites along with their chemical structures. **(ii)** Mass Spectra (MS) of the extracted metabolites along with their deuterated forms, which co-elute with the target metabolites. Deuterated metabolites were used as internal standards for quantification.



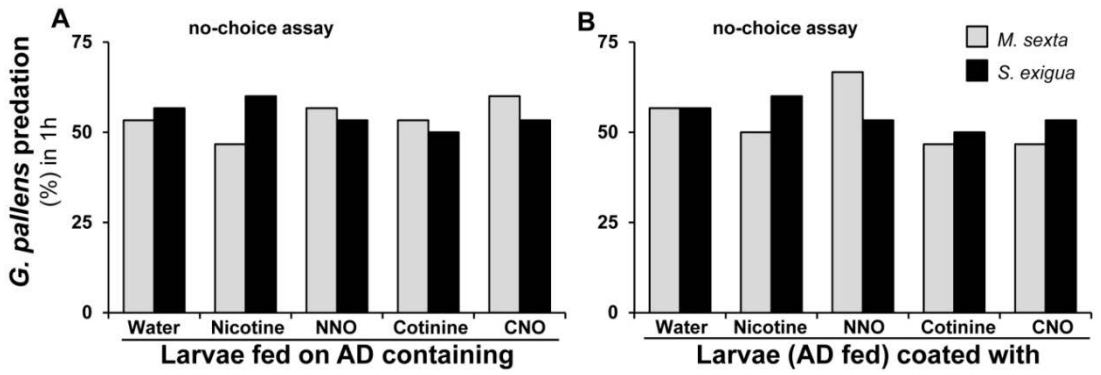
Supplementary Fig. 2 Standard curves of **(A)** nicotine **(B)** NNO **(C)** cotinine and **(D)** CNO along with equal amounts of their respective deuterated standards showing that the response of U(H)PLC/ESI-QTOF MS to all these metabolites was linear (n= 5 for each compound).



Supplementary Fig. 3 (A) Analysis showing that nicotine, NNO, cotinine or CNO are not degraded in frass over 24h period of Waldbauer assays; larval frass spiked with the respective metabolite was extracted after zero and 24h and the extracts were analysed using HPLC. Every bar represents data from 3 replicates (n= 3). **(B)** CYP4M1 [(mean± SE) $F_{4, 20}$= 9.5, P≤0.05, n= 5] and **(C)** CYP4M3 [(mean± SE) $F_{4, 20}$= 11.1, P≤0.05, n= 5] transcript levels (relative to ubiquitin) in midguts of 1st instar *M. sexta* larvae fed AD containing water (control) or 0.1% (FM) nicotine, NNO, cotinine or CNO. Asterisks above the bars indicate significant differences determined by one way ANOVA (P≤0.05).



Supplementary Fig. 4 Figure S2. S. exigua oxidizes nicotine, but M. sexta does not. U(H)PLC/ESI-QTOF-MS-based quantitative analysis of nicotine, NNO, cotinine and CNO in (a) frass (b) hemolymph and (c) headspace of third-instar M. sexta (n= 5) and S. exigua (n= 5) larvae fed N. attenuata leaves. Lower-case letters above the S. exigua bars indicate significant differences ($P \leq 0.05$) among them by one-way ANOVA. Asterisks above the M. sexta nicotine bars indicate that they are significantly different ($P \leq 0.05$) from the S. exigua nicotine bars, as determined by one-way ANOVA. Nicotine oxides were not detected in M. sexta. The detection limit of nicotine was 0.25ng and 0.5ng for cotinine, CNO and NNO; efficiency of extraction was >90% for all these compounds (Kumar et al. 2014).



Supplementary Fig. 5 *G. pallens* predation (%) (in 1h no-choice assay) on 2nd instar *M. sexta* and *S. exigua* larvae **(A)** fed AD containing water (control) or 0.1% (FM) nicotine, NNO, cotinine or CNO and **(B)** fed AD and coated with water (control) or 0.2% aqueous nicotine, NNO, cotinine or CNO. Every bar represents data from 30 larvae (n= 30).

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Chapter 6

DISSCUSSION

DISSCUSSION

In the evolutionary arms race between insects and plants, insects adapted several strategies to avoid the adverse effects of plant allelochemicals. In addition, they adopted to use plant allelochemicals to deter or poison their predators and/ or parasitoids. Numerous studies have been conducted to decode such interactions. In many instances our understanding has been limited to identification of genes involved in the detoxification processes and characterization of such genes by their products, transcription factors and detoxifying enzymes and not functional validation of the novel genes. Unavailability of transcriptome data and a reverse genetic tool to elucidate the gene function for subsequent generation of genetically modified organisms hampered the research in many insect orders including Lepidoptera.

RNAi is a recently developed technique available for functional validation of genes by reverse genetic approach. There were number of reports of gene silencing by RNAi in different orders of insects, Lepidoptera (Turner *et al.* 2006; Terenius *et al.* 2011), Diptera (Dzitoyeva *et al.* 2001) Coleoptera (Baum *et al.* 2007; Tomoyasu *et al.* 2008), Hymenoptera (Lynch & Desplan 2006) Isoptera (Zhou *et al.* 2008), and Orthoptera (Meyering-Vos & Muller 2007). The target genes selected in such studies were related to detoxification, growth, development and immunity. The success of gene silencing is highly varied according to the insect species, target tissue type and the gene function (Terenius *et al.* 2011). Silencing of a gene in a lepidopteran insect is not as efficient as coleopteran insects. Absence of RdRPs and Sid1were thought to be behind the lower silencing efficiency in lepidopterans (Gordon & Waterhouse 2007; Terenius *et al.* 2011; Zhang *et al.* 2013).

Several ways of dsRNA administration such as feeding through artificial diet, spraying dsRNA containing solution, injection of dsRNA into the hemocoel of larvae and the plant or host virus mediated delivery have been reported in lepidopteran insects (Gu & Knipple 2013; Yu *et al.* 2013). Injection of dsRNA was seen as method of choice, in many studies; however, Garbutt et al recently reported the instability of injected dsRNA molecule in the hemolymph of *M. sexta* larvae by dsRNAse. Authors also reported that

dsRNA injected into the hemolymph of German cockroach is stable for long period than dsRNA injected into the hemolymph of *M. sexta* larvae (Garbutt *et al.* 2013). There have been few reports of administration of dsRNA molecules through the artificial diet (Turner *et al.* 2006). However, because this method requires *in vitro* synthesis of large quantities of dsRNA, it is limited to the laboratory based questions and is inadequate for the ecological studies.

Administration of dsRNA through a host plant has already been reported in *H. armigera* (Mao *et al.* 2007; Mao *et al.* 2011). This was the first successful demonstration of plant mediated RNAi in lepidopteran insect species. Since we were interested in studying the ecological consequences of CYP silencing, we selected PMRi for dsRNA administration. Our results showed that stable PMRi can be a specific and robust system for gene silencing in *M. sexta.* The foremost limitation of PMRi is the long time (~10-12 months) that is required for the generation of stable transgenic *N. attenuata* plants (Gase et al. 2011). To overcome this limitation, we developed a VDPS that could be accomplished within three months. Virus mediated RNAi in insect has already been reported in nematodes (Valentine *et al.* 2007; Dubreuil *et al.* 2009). VDPS immerged as a rapid technique useful to screen many target genes in short time. In addition, we showed that VDPS as well as stable PMRi are equally efficient in gene silencing. Length of, RNAi triggering, dsRNA molecule was targeted to increase the silencing efficiency in *H. armigera*-Arabidopsis system by Mao et al. Silencing of *N. attenuata* DCLs in dsRNA expressing background plant enabled the longer dsRNA supply to the insect midgut consequently increased silencing efficiency. We have also attempted to silence the plant's dicers to increase the efficiency of PMRi. Our results were consistent with these findings showing that longer is the administered dsRNA, higher is the silencing efficiency (Kumar *et al.* 2012).

In the past, few unsuccessful RNAi experiment reports concerned the existence of RNAi pathway in lepidopteran insects. However, recent developments in RNAi research indicated the presence of core components of RNAi machinery in all insects (Gu & Knipple 2013). While there were increasing number of reports of gene silencing especially in lepidopteran insects, critics raised concerns and suggested few guidelines

for conducting RNAi experiments in lepidopteran insects (Terenius *et al.* 2011; Zhang *et al.* 2013).

We followed these guidelines and successfully demonstrated the RNAi in *M. sexta* larvae. Thus with the aid of PMRi we could gain insights to the nicotine detoxification and its adaptation by *M. sexta* larvae for the defensive use against the predators. *M. sexta* larvae have been reported to be capable of having normal growth and development while feeding on AD containing up to 1-1.5% (FM) nicotine. In 24h, 5th instar *M. sexta* larvae can ingest up to 20 mg of nicotine without displaying toxic effects and their LD50 for nicotine is the highest among all the organisms, including humans (Self *et al.* 1964a; Wink & Theile 2002).

Midgut microflora is known to be involved in digestion and detoxification of host plant compounds (Genta *et al.* 2006). However, *M. sexta* larvae's midgut microorganisms failed to detoxify ingested nicotine. Apple and Gutharie isolated the bacteria and fungi from *M. sexta* larval midgut and studied the nicotine detoxification by these isolated microorganisms. They found no evidence of nicotine metabolism. Further, Snyder et al reconfirmed these results that *M. sexta* larvae midgut microorganisms do not contribute nicotine tolerance (Apple 1961; Snyder *et al.* 1994).

Since nicotine binds to acetylcholine receptors (nAChR), nicotine tolerance by *M. sexta* larvae due the modification of nAChRs was hypothesized. Wink and Theile studied the sequence variability of α-subunit of nAChRs from nicotine sensitive an insensitive larvae of Spingidae family. There was no difference of nAChR sequences among the selected insect species, falsifying the nAChR modification hypothesis in *M. sexta* larvae (Wink 1993; Wink & Theile 2002). In addition, vertebrate's blood brain barrier has been reported to have a P-glycoprotein pump, which involved in rapid excretion of toxins and protecting the nervous system (Cordoncardo *et al.* 1989). Murray et al showed that in *M. sexta* larvae blood brain barrier possesses such a pump, which is thought to be involved in protecting CNS by avoiding nicotine that reaches CNS (Murray *et al.* 1994). However, no further studies were conducted to support this fact.

The most widely accepted theory about *M. sexta*'s nicotine tolerance has been the CYP mediated detoxification (Snyder *et al.* 1994; Glendinning 2002). However, few studies including ours contradict this line. Self et al reported that tobacco feeding larvae (hornworm, cabbage looper and budworm) do not metabolize nicotine; however, the cigarette beetle larvae which do not feed on tobacco foliage normally metabolize nicotine into cotinine (Self *et al.* 1964b). We also recently found such absence of nicotine oxides in *M. sexta*'s larval frass and hemolymph (Kumar *et al.* 2013). In addition, Morris et al reported the formation of unknown nicotine conjugates later Wink and Theile also presumed the formation of nicotine conjugates (Wink & Theile 2002). These results also support the fact that *M. sexta* does not oxidize nicotine. We tested, if the natural variants of *M. sexta* and *M. quinquemaculata*, the close relative of *M. sexta* (also known to be a specialist on solanaceous plants), oxidizes nicotine. However, the absence of nicotine oxides in hemolymph and frass of both species suggested that nicotine oxidation is not a nicotine tolerance strategy in specialist insects.

Considering the hypothesis that one or more oxidation steps of nicotine are inactive or absent in *M. sexta* individuals that we used, we supplied the intermediate substrates (NNO and cotinine) of *M. sexta*'s nicotine oxidation pathway that was proposed by Snyder et al (1994) and tested if *M. sexta* can process them to the final product. We did not find any further oxidized product (CNO). In addition, we conducted a Waldbauer assay based budgeting of these fed compounds considering that if one of these products was further detoxified or used up in primary metabolism by *M. sexta*, the excretion of the ingested metabolite would be reduced (Waldbauer 1982). However, the Waldbauer assay results showed that excretion of none of the fed compounds was notably disproportionate. It has been considered that detoxified forms are generally polar than the parent compound and in case of nicotine it was reported that oxidized products were more rapidly excreted than nicotine (Hodgson 1985; Snyder *et al.* 1994). However, no faster excretion of nicotine oxides was observed in our study, again supporting the 'no oxidation strategy' in the specialist herbivore *M. sexta.* Host-plant-defense adaption strategies of generalist and specialist insects are often different (Ali & Agrawal 2012).

Qualitative analysis of hemolymph and frass of *N. attenuata* fed *S. exigua*, *S. littoralis* and *H. virescens* showed that all of them oxidize nicotine.

Often, plant xenobiotics modification by herbivores is considered to be the detoxification; however toxicity of modified products is often not tested and compared with the parent compound. To test whether the so called detoxified products of nicotine are less toxic than nicotine, we used *S. exigua* and *M. sexta* larvae as nicotine oxidizing and not oxidizing models, respectively; we fed these oxides to larvae through the diet and measured the larval mass and mortality. Cotinine was found to be toxic to *M. sexta* by reducing the larval mass, increasing mortality and inducing the melanism in these larvae. In case of generalist insect *S. exigua* nicotine was the most detrimental, whereas NNO was the least, suggesting that NNO could be a detoxification product.

Induction of detoxification enzymes in response to xenobiotic compounds and plant allelochemicals is one of the early responses of insect herbivores. In addition to CYPs, glutathione-S-transferases, esterases, and glucosyl-transferases are the key enzymes of the insect detoxification system (Krieger *et al.* 1971; Ahmad 1983; Snyder *et al.* 1995b; Scott 1999; Ahn *et al.* 2011). Nicotine induces CYPs in *S. eridania* and *H. virescens* (Brattsten & Wilkinson 1973; Rose *et al.* 1991). Detoxification via N-oxidation of tertiary pyrrolizidine alkaloids by *Tyria jacobaeae* (Lepidoptera) is already a well-known mechanism (Ehmke *et al.* 1990). In order to test *M. sexta*'s response to nicotine, Govind et al conducted a microarray analysis of 24h old *M. sexta* larvae fed WT and irPMT (nicotine-free) *N. attenuata*. CYP6B46 was found to be downregulated in irPMT fed larvae. We validated these findings by qPCR using 24h old *M. sexta* larval midgut cDNA; we found that CYP6B46 alone was induced in response to nicotine. The induction of CYP4M1 and CYP4M3 in Snyder et al might be due to the use of different *M. sexta* strain. Since Snyder et al used 5th instar larvae and 0.75% (FM) nicotine, which is roughly seven times higher than what the host plants contain, the differences in the results could also be attributed to these factors.

Waldbauer assay based nicotine flux of CYP6B46 silenced larvae was measured. Increased excretion of nicotine through frass and reduced nicotine accumulation in

hemolymph of CYP6B46 silenced larvae suggested the involvement of CYP6B46 in passing nicotine from midgut into hemolymph. We suggest that Waldbauer assays are highly useful to measure the amount of a metabolized compound, especially when the detoxification product is not known.

For *Ms*CYP6B46's function discovery, we adapted 'ask the eco-system approach'. Predatory native spiders of Utah clearly preferred to prey on CYP6B46 silenced *M. sexta* larvae like nicotine-free larvae. Our work demonstrated a function of *Ms*CYP6B46 in allowing nicotine to pass through the larvae's digestive system to hemolymph by an unknown mechanism, which in turn provides a means of externalizing nicotine into the larvae's headspace. Predatory wolf spiders, which are known to possess a well-developed olfaction system were found to be deterred by the volatile nicotine exhaled by larvae (Persons *et al.* 2001). We demonstrated that specialist herbivore *M. sexta* not only adapted to nicotine but also exploit it for its own purpose. Similar type of interaction is also seen in other insect species including larvae of several zygaenid moths (Zygaenidae, Lepidoptera) feeding on the plants that contain cyanogenic glucosides (Zagrobelny & Moller 2011). These larvae synthesize hydrogen cyanide using host plant metabolite cyanogenic glucosides, which is toxic to the predators.

Plants are known to release volatiles in response to insect attack to attract predators (Kessler & Baldwin 2001). I infer that specialist herbivore *M. sexta* copied such a trait to deter their predators. It will be interesting to study host plant volatile mediated attraction and deterrence by other lepidopteran herbivores.

Considering the catalytic functions of insect CYPs, it is unlikely that CYP6B46 itself acts as a nicotine-pump. However, it could be part of a multicomponent system and is involved in the activation of actual transporters. Alternatively, the midgut based CYP6B46 may convert nicotine to an 'easy to pump across the midgut form', which is short lived and rapidly reverts back to nicotine after entering hemolymph, as proposed by Murray et al (Murray *et al.* 1994).

Host plant defense adaption strategies of generalist and specialist insects are often different (Vogel *et al.* 2007; Ali & Agrawal 2012). Host plants' xenobiotics are less toxic to the specialist insects than generalist insects. The most important result of our study showed that oxidized forms of nicotine are not deterrent to the spiders. This could be another front where the specialist *M. sexta* benefits at the ecological level by not detoxifying nicotine, whereas the generalists oxidize nicotine (probably, to gain the physiological comfort), at the cost of their survival in the spider-rich native habitat **(Fig. 5)**. On the other hand, cotinine, the chief nicotine oxide, was found to be more harmful to the herbivore, than nicotine. Thus it is likely that the enhanced toxicity of nicotine to the generalist is due to its oxidation to cotinine and the host plant benefits form this mechanism. Why such a harmful mechanism exists in the generalists is a question of future research: Based on the general alkaloid responsive induction behavior of CYP6B46 in *M. sexta* (even after which alkaloid oxidation was not observed), it can be postulated that the detoxification systems in the generalist herbivores are also a subject to a general induction by various xenobiotics; in case of certain xenobiotics they succeed in detoxification, whereas in case of the ones like nicotine they end up in converting the protoxins to the toxin.

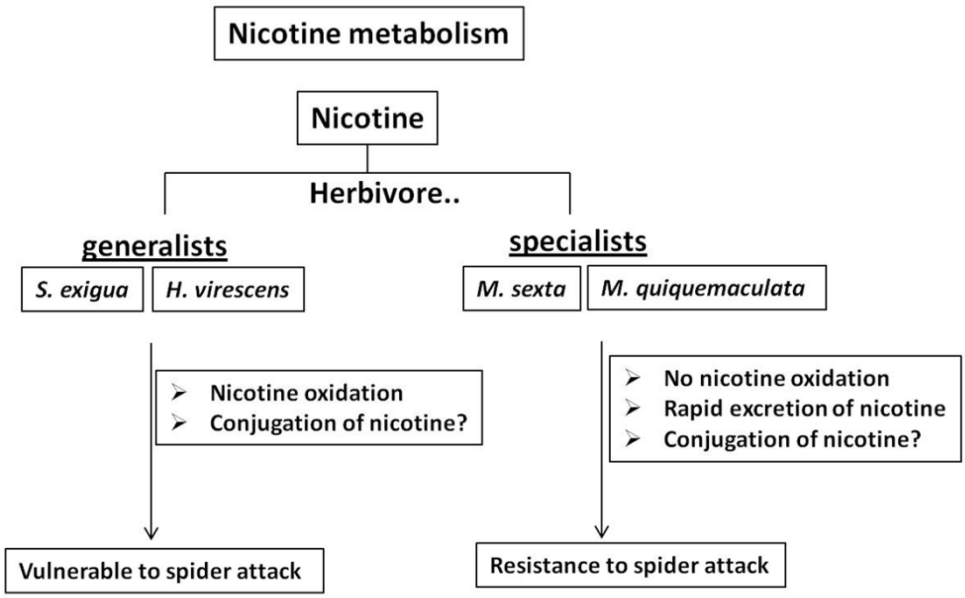

Fig. 5 Nicotine metabolism in various herbivores and its ecological consequences.

Nicotine detoxification strategy of generalist and specialist herbivores is different. Generalist insects detoxify nicotine by oxidation, however become vulnerable to the spider attack. Specialist insects rapidly excrete ingested nicotine without oxidation and by exhaling a part of it, they become resistant to the spider attack.

We also demonstrated that another abundant predator, the big eyed bug was not deterred by the nicotine feeding larvae. However, it is plausible that the herbivores co-opt unmetabolized xenobiotics against one predator and the metabolized ones against another. Therefore, we tested if various nicotine oxides confer the herbivores an advantage against *G. pallens* that has a diurnal preying time (as against the nocturnal spiders) and a different prey selection and feeding behavior than the spider. However, big eyed bugs were also found to be insensitive to nicotine oxides like nicotine.

Larvae's toxic nicotine-rich breath serves as a volatile signal against the predatory spiders and this trait is predator specific, since volatile nicotine does not deter big eyed bugs. Whether *Ms*CYP6B46 is also responsible for the differential survival of endoparasitoids (Thorpe & Barbosa 1986) is an intriguing possibility deserving further study. It will be interesting to see if the other nicotine-tolerant near relatives of *M. sexta* that normally feed on non-nicotine containing host plants have evolved similarly functioning orthologs of CYP6B46.

We conclude that *M. sexta* larvae do not oxidize nicotine. Nicotine oxides do not provide physiological (rapid excretion) and ecological advantage (predation resistance) to *M. sexta* larvae. In fact cotinine is toxic to *M. sexta* larvae. I infer that by keeping nicotine unmetabolized, the specialist herbivore avoids the expensive oxidation and also benefits against the spiders; whereas, oxidation could be a general and spontaneous response of the generalist insects that renders them susceptible against the predators. This work highlights the importance of host chemistry in the long-debated generalist-specialist paradigm and support that the natural enemies are significant selective forces in the evolution of herbivore- host plant relationship.

SUMMARY

Insects belong to the most diverse group of animals on the planet and they are found in nearly all environments; thus, they have developed a myriad of adaptations to live and evolve. *Manduca sexta,* a specialist lepidopteran herbivore of *Nicotiana attenuata,* has developed a remarkable adaptation to tolerate a toxic alkaloid 'nicotine' that it ingests from its host plant *Nicotiana attenuata*. In fact, *M. sexta* is a highest nicotine tolerating organism known. The mechanism of nicotine tolerance in *M. sexta* larvae is not completely known. In this work I used a reverse genetics based approach to silence *M. sexta*'s genes involved nicotine tolerance to understand their role and ecological consequences of nicotine adaptation by this insect.

I developed a transgenic *N. attenuata* plant having stable and transient expression of *Ms*CYP6B46 dsRNA. Larvae feeding on such plants continuously ingest this dsRNA, which enables a specific and robust means of silencing the target gene CYP6B46 in *M. sexta* midgut. Survivorship of CYP6B46-silenced *M. sexta* larvae was found to be lowered, similar to that of low-nicotine-foliage feeding larvae in the field (Great Basin desert, Utah). While investigating the reasons for the reduced survivorship, I identified a native wolf spider that preferred to prey on nicotine-free and CYP6B46-silenced larvae, compared to the wild type *N. attenuata* feeding control larvae. Further, I found that CYP6B46-silenced larva is impaired in passing nicotine from midgut into hemolymph, thereby reducing the availability of nicotine for emission through spiracles and hence become susceptible to spider attack.

Although detoxification of nicotine by oxidation was reported in literature, I did not detect any of oxidized form of nicotine. However, a generalist lepidopteran herbivore of *N. attenuata*, *Spodoptera exigua*, was found to oxidize nicotine. Spider predation assay using oxidized forms of nicotine suggests that spider deterrence could be attributed only to nicotine and not to the oxidized forms of nicotine, indicating that modification of nicotine increased the larval vulnerability to spider predation. In addition, there are no physiological advantages of nicotine oxides in terms of rapid excretion and mass gain of *M. sexta* larvae. I infer that nicotine detoxification in *M. sexta* by oxidation of nicotine is a physiologically and ecologically expensive strategy. I conclude that 'use of unmetabolized nicotine against spiders' is a specialty of *M. sexta* and CYP6B46 mediates its flux of nicotine from midgut to hemolymph for subsequent volatilization to deter spiders.

ZUSAMMENFASSUNG

Die Aufdeckung von *Manduca sexta*s Nikotinmetabolismus und seine ökologischen Konsequenzen analysiert durch auf pflanzenvermittelterRNAi basierter reverser Genetik

Insekten gehören zu der facettenreichsten Gruppe im Tierreich und sind in nahezu allen Lebensräumen vertreten. Aus diesem Grund haben sie auch eine Unzahl an Anpassungen vollzogen, um ihr Überleben zu sichern und sich zu entwickeln. Der Tabakschwärmer (*Manduca sexta*), ein zur Ordnung der Schmetterlinge gehörender Pflanzenfresser, der sich als Spezialist von *Nicotiana attenuata* ernährt, hat eine besondere Anpassung entwickelt, um das ansonsten giftige Alkaloid Nikotin, das er von seiner Wirtspflanze *N. attenuata* aufnimmt, zu tolerieren. Bemerkenswert ist hierbei, dass *M. sexta* von allen derzeit bekannten Lebewesen, dasjenige mit der höchsten Toleranzschwelle für Nikotin ist. Allerdings ist der Mechanismus der Nikotintoleranz in *M. sexta* noch nicht vollständig aufgeklärt. In dieser Arbeit habe ich als methodischen Ansatz reverse Genetik genutzt, um die Rolle von *M. sexta* Genen, die für die Nikotintoleranz verantwortlich sind, zu untersuchen und die ökologischen Auswirkungen der Nikotinadaptation von *M.sexta* zu verstehen.

Dazu kreierte ich eine transgene Wirtspflanze mit stabiler und transienter Expression von *Ms*CYP6B46 dsRNA. Durch Abfütterung der Larven auf diesen Pflanzen wird die dsRNA kontinuierlich aufgenommen, was ein spezifisches und robustes Mittel zum Gen-Silencing des Zielgenes CYP6B46 im Mitteldarm von *M. sexta* darstellt. Die Überlebensrate von Larven mit stillgelegtem CYB6B46 ist signifikant geringer, vergleichbar mit der von Larven, die im Feld (Great Basin Wüste, Utah), mit Blättern mit einem geringen Nikotingehalt gefüttert wurden. Bei der Untersuchung der Gründe für die geringere Überlebensrate fand ich heraus, dass die einheimische Wolfsspinne Larven mit niedrigem Nikotingehalt und stillgelegtem CYP6B46 bevorzugt, verglichen mit der mit Blättern von *N.attenuata* Wildtyp gefütterten Kontrollarve. Darüber hinaus fand ich heraus, dass in Raupen, deren CYP6B46 stillgelegt war, der Übergang von Nikotin vom Mitteldarm in die Hämolymphe beeinträchtigt war, was dazu führt, dass Nikotin nicht mehr durch die Stigmata abgegeben werden kann und dadurch die Larve anfälliger für Attacken durch räuberische Spinnen wird.

Obgleich die Nikotinentgiftung durch Oxidation bereits in der Literatur beschrieben wurde, konnte ich keine oxidierte Form nachweisen. Allerdings wurde bei einem anderem Vertreter der pflanzenfressenden Schmetterlinge, *Spodoptera exigua,* einem Generalisten, Nikotinoxid gefunden. Spinnenräuberassays mit oxidierten Formen von Nikotin legen nahe, dass die Spinnenabschreckung nur durch Nikotin wirksam ist und nicht den Oxidationsprodukten zugeschrieben werden kann, was darauf hindeutet, dass Nikotinmodifikationen die Anfälligkeit der Raupen für Prädation durch die Spinne erhöhen. Zudem gibt es keine physiologischen Vorteile durch Nikotinoxide im Hinblick auf schnelle Ausscheidung oder Gewichtszunahme der *M. sexta* Larve. Daraus kann ich ableiten, dass die Entgiftung durch Oxidation von Nikotin in *M. sexta*, eine physiologisch und ökologisch teure Strategie ist. Zusammenfassend schließe ich daraus, dass die Verwendung von nicht umgewandeltem Nikotin eine Besonderheit von *M. sexta* ist, und dass CYP6B46 den Transfer von Nikotin vom Mitteldarm zur Hämolymphe für eine nachfolgende Verflüchtigung vermittelt, um Spinnen abzuschrecken.

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EIGENSTÄNDIGKEITSERKLÄRUNG

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, daß ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

Jena, den 20. Dezember 2013

Pavan Kumar

Erklärung über laufende und frühere Promotionsverfahren

Hiermit erkläre ich, dass ich keine weiteren Promotionsverfahren begonnen oder früher laufen hatte. Das Promotionsverfahren an der Biologisch-Pharmazeutischen Fakultät ist mein erstes Promotionsverfahren überhaupt.

Jena, den 20. Dezember 2013

Pavan Kumar

CURRICULUM VITAE

Pavan Kumar
Department of Molecular Ecology
Max-Planck Institute for Chemical Ecology
Hans-Knöll-Straße 8
D-07745 Jena, Germany
+49-17693120227
+49-3641-571123
pkumar@ice.mpg.de

Personal profile

Date of birth**:**	17th September 1982
Birth place:	Barur, India
Sex**:**	Male
Nationality**:**	Indian
Permanent address**:**	Post: Barur, Tq&Dt: Bidar-585401 Karnataka state- India Ph: 91-8482 200081 Email: pavankumar.sk@gmail.com

Educational qualifications

- **Master of Science (MSc)** in Biotechnology from Department of Biotechnology, Gulbarga University, Gulbarga India April-2005

- **Bachelor of Science (BSc)** in Biotechnology, Chemistry and Zoology at Karnataka college, Gulbarga University Bidar, India April-2003

Research experience

- As a project assistant at Plant Molecular Biology Unit, National Chemical Laboratory, Pune, India September 2005- March 2008

  Project: Identification and characterization of plant proteinase-inhibitor insensitive lepidopteran insect *Helicoverpa armigera* midgut proteases

- As a senior research fellow at National Center for Cell Science, Pune India February 2008- February 2009

  Project: Elucidation of role of matrix associated binding protein SMAR1 in transcriptional regulation of genes associated with cervical cancer

Publications

1. Spatial and temporal expression patterns of diverse Pin-II genes in *Capsicum annuum* Linn., Tamhane VA, Giri AP**, Kumar P**, Gupta VS (2009) ***Gene*** 442:1-2 (88-98)
2. Tobacco Rattle Virus vector: A rapid and transient means of silencing *Manduca sexta* genes by plant mediated RNA interference. **Kumar P**, Pandit SS, Baldwin IT T (2012) ***PLoS One***, 7(2), e31347
3. A natural history driven, plant mediated RNAi based study reveals CYP6B46's role in a nicotine-mediated anti-predator herbivore defense. **Kumar P**, Pandit SS, Steppuhn A, and Baldwin IT (2013) Proceedings of the National Academy of Sciences (in press)
4. Differences in nicotine metabolism of two Nicotiana attenuata herbivores render them differentially susceptible to a common native predator. **Kumar P**, Rathi P, Schöttner M, Baldwin IT and Pandit SS. Communicated to Ecology letters

Project work during graduation

- Identification and characterization of glutamate decarboxylase and its gene in sugarcane (*Saccharum officinarum* .L*)* var.co.740 under salt stress', submitted to Department of Biotechnology, Gulbarga University Gulbarga India as partial fulfillment for MSc degree guided by Prof. G. R. Naik in 2005
- 'Bio processing of organic wastes for improved qunto-quality production bio-gas as an efficient source of fuel', submitted to Department of Biotechnology, Karnataka College Bidar, India as a partial fulfillment for BSc degree, guided by Dr. Vijay Kumar Biradar in 2003

Language proficiency

English,
Hindi, Kannada, Telugu, Marathi (Indian languages) and
German (Qualified B1 level from EURSIA institute Berlin, Germany 2009)

Oral presentations

- Probing insect response to nicotine by plant mediated RNA interference at 10th IMPRS symposium, Dornburg Germany on 12th February 2011
- Detoxification of diterpenoid glycosides by *M. sexta* at Castle Ringberg, Kreuth Germany 9th November 2009

Poster presentations

- Kumar P, Baldwin IT and Pandit S**,** *Manduca sexta* larvae co-opt plant defense metabolite nicotine to deter spiders, 12th IMPRS Symposium, MPI for Chemical Ecology, Jena, Germany April 2013
- Pandit S, Kumar P, Kessler D, and Baldwin IT*,* Plant mediated RNA interference for the reverse genetics of a native insect herbivore in the field, Scientific advisory board meeting, MPI for Chemical Ecology, Jena, Germany, October October 2012
- Kumar P, Pandit S, and Baldwin IT *Manduca sexta*'s nicotine metabolism is influenced by the environment. 11th IMPRS symposium, MPI for Chemical Ecology, Dornburg, Germany, February 2012
- Kumar P, Poosakkannu A, Pandit S, Bozorov T, Kröber W, Kessler D, Schoetner M and Baldwin IT, Plant mediated RNA interference for insect gene silencing. Scientific advisory board meeting, MPI for Chemical Ecology, Jena, Germany, October 2010
- Kumar P, Sweet poison sours caterpillar's life. 9th IMPRS symposium, MPI for Chemical Ecology, Dornburg, Germany, February 2010

Conference

- Poster presented on **"**Identification and characterization of glutamate decarboxylase and its gene in sugarcane (*Saccharum officinarum* .L*)* var.co.740 under salt stress" Babu G, Kumar P and Naik G R, at International conference on **"Plant genomics on biotechnology: Challenges and opportunities**" October 26-28, 2005 at Indira Gandhi Agricultural University, Raipur, India

Workshop

- Analysis of small molecules 23rd- 28th January 2012 at MPI for Chemical Ecology, Jena, Germany
- Advanced course on analytical chemistry 22nd- 26th February 2011 at MPI for Biogeochemistry, Jena, Germany
- Life after PhD; funding gates at MPI for Chemical Ecology, March 2011, Jena, Germany
- Advanced molecular cloning 7th-11th November 2011 at MPI for Chemical Ecology, Jena, Germany

- Proteomic insights into plant-insect interactions sponsored by Max-Planck society-India partnership program at National Chemical Laboratory (NCL), Pune 12th-15th December 2006, India
- Basic techniques in molecular biology and immunology at Institute of Biosciences and Molecular Biology (IBMB) and Bangalore Genei Pvt. Ltd., Bangalore 2005, India

Awards

1. Gold medal for BSc on 22nd annual convocation of Gulbarga University Gulbarga, India 3rd January 2004
2. R. V. Bidap Higher Education scholarship of year 2004 from Karnataka College (K.R.E.S Society) Bidar, India to pursue master degree
3. Merit student fellowship during MSc from Gulbarga University, Gulbarga, India in the academic year 2004-05
4. Rank student in MSc Biotechnology 2005
5. DAAD doctoral fellowship to pursue PhD degree in Germany 2009

PUBLICATIONS, TALKS AND POSTERS

❖ Publications

- ❖ Spatial and temporal expression patterns of diverse Pin-II genes in *Capsicum annuum* Linn., Tamhane VA, Giri AP**,** **Kumar P**, Gupta VS (2009) ***Gene*** 442:1-2 (88-98)
- ❖ Tobacco Rattle Virus vector: A rapid and transient means of silencing *Manduca sexta* genes by plant mediated RNA interference. **Kumar P**, Pandit SS, Baldwin IT T (2012) ***PLoS One***, 7(2), e31347
- ❖ A natural history driven, plant mediated RNAi based study reveals CYP6B46's role in a nicotine-mediated anti-predator herbivore defense. **Kumar P**, Pandit SS, Steppuhn A, and Baldwin IT (2013) Proceedings of the National Academy of Sciences (in press)
- ❖ Differences in nicotine metabolism of two Nicotiana attenuata herbivores render them differentially susceptible to a common native predator. **Kumar P**, Rathi P, Schöttner M, Baldwin IT and Pandit SS. Communicated to Ecology letters

❖ Talks

- Probing insect response to nicotine by plant mediated RNA interference at 10th IMPRS symposium, Dornburg Germany on 12th February 2011
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- Life after PhD; funding gates at MPI for Chemical Ecology, March 2011, Jena, Germany
- Advanced molecular cloning 7th-11th November 2011 at MPI for Chemical Ecology, Jena, Germany
- Proteomic insights into plant-insect interactions sponsored by Max-Planck society-India partnership program at National Chemical Laboratory (NCL), Pune 12th-15th December 2006, India
- Basic techniques in molecular biology and immunology at Institute of Biosciences and Molecular Biology (IBMB) and Bangalore Genei Pvt. Ltd., Bangalore 2005, India

❖ Public relationship activity

- Actively participated in Lange nacht der wichenschaft at MPI for Chemical Ecology, Jena, Germany 2011 by exhibiting different stages of *M. sexta* life cycle to visitors and encouraged kids to draw the life stages of *M. sexta*
- Educated to undergraduate students at Karnataka college, Bidar, India by delivering a talk on 'Plant mediated RNAi for insect gene silencing' on 9th February 2012

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Pavan Kumar